IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
KIM *et al.*
Appl. No.: 10/646,145
Filed: August 22, 2003
For: **Composition Comprising the Extract of Actinidia Arguta and Related Species for the Prevention and Treatment of Allergic Disease and Non-Allergic Inflammatory Disease**

Confirmation No.: 8727
Art Unit: 1627
Examiner: SOROUSH, Layla
Atty. Docket: 2298.0140001/TJS/M-N/MSS

## Declaration of Sunyoung Kim, D. Phil., Under 37 C.F.R. § 1.132

***Mail Stop Amendment***

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned, Sunyoung Kim, D. Phil., residing at 390 Egok-ri Soheul-eup Pocheon-si, Gyeong Gi-Do, Korea 487-821, declares and states as follows:

1. I am a professor of the Seoul National University. I am also a major inventor listed in the above-captioned patent application. My credentials are provided in the *Curriculum Vitae* that is attached to this declaration as Exhibit A. I received my doctoral degree in the field of molecular genetics, from the Department of Biochemistry, University of Oxford. Thereafter, I did my postdoctoral work in Dr. David Baltimore's laboratory, that time at the Whitehead Institute, MIT. Before I came to Seoul National University in 1992, I ran my own laboratory as an assistant professor of medicine, Harvard University. As seen from my attached *Curriculum Vitae*, I have extensively investigated the therapeutic uses of various botanical products as well as many biologicals, and have particular expertise in isolating extracts for the treatment of various

inflammatory diseases. I have published several papers related to the development of fruit extracts for disease treatment.

2. I have reviewed the outstanding Office Action in U.S. Appl. No. 10/646,145, as well as the references cited therein. It is my understanding that Hunder *et al., Arthritis & Rheumatism 17(6)*:955-963 (1974) ("Hunder"), which was cited against the `145 application, is considered by the Examiner to suggest that "[t]here is an increase of IgE in patients with rheumatoid arthritis" and that "[t]reatment of rheumatoid arthritis would result in the decrease of IgE in patients with rheumatoid arthritis." *See* Office Action at page 6.

3. However, Hunder clearly states in their abstract that "no significant correlation was found between serum IgE concentration and severity of RA." *See* abstract of Hunder at page 955. Any scientist of ordinary skill in the field would interpret, based on scientifically acceptable statistical standards, the statement "no significant correlation" to mean that there was no statistically significant correlation between serum IgE concentration and severity of RA, and that any variations of serum IgE concentration seen in patients with varying degrees of RA symptoms occurred by chance alone. *See* "Quantitative Techniques," *NIST/SEMATECH e-Handbook of Statistical Methods*: Sections 1.3.5 – 1.3.5.3, National Institute of Standards and Technology - U.S. Commerce Department (2010) (enclosed herein as Exhibit B) ("NIST") supports this interpretation and defines the "hypothesis test," which includes the two-sample *t*-Test statistical approach used by Hunder, as follows:

> [A] hypothesis test attempts to refute a specific claim about a population parameter based on the sample data...To reject a hypothesis is to conclude that it is false. However, to accept a hypothesis does not mean

> that it is true, only that we do not have evidence to believe otherwise. This hypothesis tests are usually stated in terms of both a condition that is ***doubted (null hypothesis)*** and a condition that is ***believed (alternative hypothesis)***... Statistical significance simply means that we reject the null hypothesis.

*See* NIST at Section 1.3.5, page 245 (emphasis added).

Therefore, Hunder in view of NIST indicates a null (doubted) hypothesis that variations of serum IgE concentration seen in patients with varying degrees of RA symptoms occurred by chance alone and implies an alternative (believed) hypothesis that a correlation between serum IgE concentration and severity of RA exists. Given that Hunder found no significant correlation between serum IgE concentration and the severity of RA and based upon the teachings of NIST, the alternative (believed) hypothesis is rejected, and the null (doubted) hypothesis of "variations of serum IgE concentration seen in patients with varying degrees of RA symptoms occurred by chance alone" must be accepted.

NIST further teaches that "[t]wo-sample *t*-tests can be used to answer... Is process 1 ***equivalent*** to process 2?" *See* NIST at Section 1.3.5.3, page 260 (emphasis added). In view of NIST, the lack of a statistically significant correlation between the severity of RA and increased serum IgE concentration as determined by Hunder via the two-sample *t*-test statistical approach further implies that the severity of RA ***does not equate with*** increased serum IgE concentration and that there was ***no correlation between*** increased serum IgE and the severity of RA.

Aihara, Y., Jahromi, B., Yassari, R., Savama, T., and Macdonald, R. *Neurosurgery 52*: 661-667 (2003) (enclosed herein as Exhibit C) ("Aihara") further

supports the notion that "***no significant correlation***" is equivalent to "***no correlation***" in the following statement:

> There was ***no significant correlation*** between arterial cGMP contents and the severity of vasospasm. Conclusion: DETA/NO did not prevent vasospasm. There was ***no correlation*** between the severity of vasospasm and cyclic adenosine monophosphate and cGMP levels in the cerebral arteries.

*See* Airhara abstract (emphasis added).

The statement in Hunder that "***[n]o significant correlation*** was found between serum IgE concentration and the severity of RA" not only implies "***no correlation***" and "***not equivalent to***" but also implies that the severity of RA "***does not depend on***" serum IgE concentration and that the severity of RA has "***no association***" with serum IgE concentration. Nguyenkim, J. and DeAngelis, G. *The Journal of Neuroscience 23*: 7117-7128 (2003) (enclosed herein as Exhibit D) ("Nguyenkim") supports the aforementioned implied interpretations of "no significant correlation" in the following statement:

> We find ***no significant correlation*** between these variables ($r = 0.02$; $p = 0.89$), indicating that tilt tuning ***does not depend on*** asymmetric surround effects.

*See* Nguyenkim at page 7126, lines 29-31 (emphasis added).

Halawaty, S., ElKattan, E., Azab, H., ElGhamry, N., and Al-Inany, H. *J. Obstet. Gynaecol. Can. 32*: 687-690 (2010) (enclosed herein as Exhibit D) ("Halawaty") further supports these interpretations of "no significant correlation" in the following statement:

> There was ***no significant difference*** between the two groups in mean age, levels of serum AMH, serum FSH, FBG, 2 hr PP, or AFC. Ovarian volume was significantly lower in obese women (3.7 ± 0.8 ml) than in non-obese women (6.6 ± 0.4 ml) ($P = 0.03$). There was ***no significant correlation*** between BMI and serum AMH, serum FSH, FBS, or 2 hr PP. Conclusion: Obesity has ***no association*** with levels of serum FSH, AMH,

> blood glucose, or AFC indicating that obesity is unlikely to affect ovarian reserve in the perimenopausal age group.

*See* Halawaty abstract at page 687 (emphasis added).

In summary, the statement in Hunder that "***[n]o significant correlation*** was found between serum IgE concentration and the severity of RA" implies the acceptance of the "no correlation" null hypothesis and should be interpreted to mean the following: the severity of RA ***does not equate with*** increased serum IgE concentration, there was ***no correlation*** between serum IgE concentration and the severity of RA, the severity of RA ***does not depend on*** serum IgE concentration, and the severity of RA has ***no association*** with serum IgE concentration.

4. The fact that Hunder reports no change in serum IgE concentrations among RA patients with low to high levels of RA pathology indicates that RA therapies would not alter serum IgE levels. That is, a scientist would expect, based on the teachings of Hunder, that reducing the symptoms of a patient with advanced RA disease to a level seen in a patient with mild RA disease through RA therapies would have no impact on serum IgE levels. Hunder supports this line of reasoning in their statement that "increased IgE in RA may be the result of a general immune response seen in this disease." *See* abstract of Hunder at page 955. Furthermore, Hunder reports that IgE levels were not elevated in the synovial fluid of RA patients. Synovial fluid is the fluid found in the cavities of most joints in the body. Because synovial joints are the principal site of RA pathology, the lack of elevated IgE in the synovial fluid of RA patients indicates that the IgE class of antibodies are not principally involved in RA pathology. The lack of elevated IgE levels in arthritic joints and the lack of a correlation between

serum IgE levels and RA disease severity is not surprising, because it has been well established that the IgE class of antibodies is primarily associated with an allergic response, while RA is well-known to be an autoimmune disorder, not an allergic disease.

5. Hunder teaches that "[s]erum and synovial fluid levels of immunoglobulin E (IgE) were significantly increased in patients with rheumatoid arthritis (RA) but were normal in degenerative arthritis (DJD) . . . [T]he increased IgE in RA may be the result of a general immune response seen in this disease." *See* abstract of Hunder at page 955. This statement in Hunder refers to the increased levels of IgE in RA patients compared to degenerative arthritis (DJD) patients and does not refer to a comparison of serum IgE levels among patients with varying degrees of severity of RA or between RA patients and healthy patients. Table 1 of Hunder indicates a significant increase in serum IgE concentrations between patients with RA and DJD but does not report a significant increase in serum IgE concentrations between RA patients and normal patients. Table 1 of Hunder further indicates that the values for serum IgE concentrations in normal patients were derived from a previously published study. A statistical comparison of serum IgE levels between normal patients and RA patients may have been omitted by Hunder, because the comparison of values between two studies often prevents the establishment of a valid statistical correlation due to differences in experimental techniques, experimental conditions, control groups, and other potential variables between the two studies. For the reasons mentioned above, the teachings of Hunder do not exclude the likely possibility that the lower serum IgE levels in DJD patients compared to RA patients were the result of or caused by the DJD disease state. Therefore, a scientist of ordinary skill in the art would at most interpret the data

disclosed in Table 1 of Hunder to suggest a correlation between serum IgE levels and different types of arthritic disease (*i.e.*, RA and DJD) and would not erroneously interpret the data disclosed in Table 1 to suggest a statistical difference or correlation between serum IgE levels of healthy patients and RA patients. As stated above, the absence of a statistically significant correlation concedes the null hypothesis of no correlation.

6. The general consensus in the scientific community at the time of the filing of this patent application was that a role(s) for IgE antibodies in RA pathology was at most negligible or non-existent. Furthermore, there are multiple scientific studies suggesting that if arthritic patient cohorts are carefully and appropriately compared to non-arthritic patient control cohorts (for example, considering such factors as age and sex) then no difference in serum IgE levels exist between arthritic and non-arthritic patient cohorts. This general consensus regarding the lack of a correlation between IgE levels and RA disease is exemplified by Marcolongo, R. and Marsili, C. *Z. Immun.-Forsch. Bd. 148*:S 285-290 (1975) (Exhibit E) ("Marcolongo"); Peskett, S. *et al. J. Rheumatol. 8*: 321-324 (1981) (Exhibit F) ("Peskett"); and O'Driscoll, B. *et al. Clinical Allergy 15*: 547-553 (1985) (Exhibit G) ("O'Driscoll"). Marcolongo, Peskett, and O'Driscoll all report that total serum IgE levels in RA patient cohorts were not elevated in relation to control cohorts. Marcolongo states, "[n]o correlation of . . . IgE values . . . with the activity and the duration of the rheumatoid arthritis was observed." *See* abstract of Marcolongo at page 285. Additionally, Marcolongo suggests that "the role and the importance of . . . IgE immunoglobulins may be excluded or considered to be negligible in . . . immunological response related to rheumatoid arthritis." *See*

Marcolongo at page 288. Peskett examined total IgE levels and pollen specific IgE in the sera of patients with adult rheumatoid arthritis and states in the abstract that "[t]he incidence of IgG and IgE antibodies to pollen appeared to be low and the ***geometric mean total IgE was low***" in this patient population compared to those of a control group. *See* abstract of Peskett (emphasis added). Therefore, Peskett directly contradicts the Examiner's interpretation of Hunder regarding total IgE levels in RA patients.

O'Driscoll directly addresses the studies of Peskett and Hunder. O'Driscoll confirmed the findings of Peskett and states that they "[d]id not find an elevated serum level of IgE in RA patients compared with controls" and that their results "[w]ere similar to those of Peskett *et al.* who found that ***total serum IgE in adults with RA was similar to (but slightly lower than) that of normal controls***." *See* O'Driscoll at page 552, paragraph 5 (emphasis added). O'Driscoll states that the findings of Hunder were flawed, because the authors of Hunder "[u]sed controls which were not matched for age and sex (it has subsequently been shown that serum IgE levels diminish with advancing age, especially above the age of 65 years) [18]." *See* O'Driscoll at page 552, paragraph 5. O'Driscoll further elaborates on the flaws of the Hunder study in the following statement:

> The high mean IgE among the RA patients was largely attributable to a small number of patients with an extremely high serum IgE. The median serum IgE was not reported... and as serum IgE values are not normally distributed, the median value is probably more important than the mean.

*See* O'Driscoll at page 552, paragraph 5.

In summary, Marcolongo, O'Driscoll, and Peskett reflect the general consensus in the scientific community at the time of the filing of the present application that a role for IgE antibodies in RA pathology was, at most, negligible or non-existent. O'Driscoll

indicates that the Hunder study was flawed in both selection of controls and in the data analysis of serum IgE values in the various cohorts.

7. It is my understanding that the Examiner cited Myers *et al.*, *Arthritis & Rheumatism 43(12)*:2687-2693 (2000) ("Myers"), against U.S. Appl. No. 10/646,145 and views this paper as suggesting that treatment of rheumatoid arthritis (RA) would result in the decrease of IgG1 and increase of IgG2a in patients. The Examiner quoted the following from Myers to support her assumption:

> Compared with wild-type controls, COX-1-/- mice exhibited a slight increase in IgG2a antibody production and a slight decrease in IgG1 antibodies. Conversely, COX-2-/- mice exhibited significantly depressed levels of both IgG1 and IgG2 antibodies (Table 1).

*See* Myers at 2690.

The Examiner's argument that treatment of rheumatoid arthritis (RA) would result in the decrease of IgG1 and increase of IgG2a in patients is a misinterpretation of Myers. Myers examined whether or not the COX-1 and COX-2 genes were required for the development of arthritis in a murine model system. Myers reports that mice deficient in COX-1 (COX-1-/-) developed arthritis and that COX-1-/- was required for the development of arthritis following vaccination with the self-antigen CII. Myers further reported that a slight increase in CII-specific IgG2a antibody production and a slight decrease in CII-specific IgG1 antibodies were seen in the COX-1-/- mice compared to arthritic control mice.

8. The Examiner's interpretation of Myers' results regarding COX-1-/- mice assumes that there were significant differences in IgG2a and IgG1 antibody levels in comparison to arthritic WT control mice. However, the differences in IgG2a and IgG1

antibody levels between COX-1-/- mice and control mice were *not statistically significant.* Therefore, no or little correlation between changes in IgG2a or IgG1 antibody levels and the presence or absence of the COX-1-/- gene was found.

9. The Examiner failed to notice in the Myers study that antibody levels of arthritic COX-1-/- mice were compared to antibody levels of arthritic control mice. Myers does not disclose any data or data comparisons involving a non-arthritic control mouse population. As a result, a scientist cannot compare the changes in IgG1 and IgG2a levels of COX-1-/- mice to those of mice that have no arthritic disease or reduced arthritic disease. Therefore any non-statistically significant changes in CII-specific IgG1 and IgG2a antibody levels reported in COX-1-/- mice in relation to WT CII vaccinated mice neither predict nor necessarily reflect any changes that would occur following RA treatment.

10. Myers does not account for the effects of the COX-1 deletion itself on the immune response or antibody production. A scientist would expect that the genetic deletion of COX-1, which is the target of anti-inflammatory NSAID medications such as aspirin, would have a major impact on the production of many different cytokines and antibodies. To account for such a broad range of immune changes resulting from COX-1 deficiency, Myers should have used a non-CII vaccinated COX-1-/- and non-CII vaccinated WT control populations, which they did not. Therefore, one cannot exactly know, based solely on the disclosure of Myers, whether alterations observed in the immune response of COX-1-/- mice are the result of COX-1 deficiency or reflect the induction of the arthritic disease state resulting from CII vaccination. Therefore, the

teachings of Myers do not enable one of ordinary skill in the art to predict the effects of RA therapy on serum IgG1 and IgG2a levels in a clinical setting.

11. Myers presents evidence that COX-2-/- mice exhibited a reduced incidence of arthritis in response to the CII vaccine. Compared to the arthritic wild-type control mice, COX-2-/- mice exhibited significantly depressed levels of **both** CII-specific IgG1 and IgG2a antibodies. However, these findings are irrelevant to the claimed invention, because the present invention does not relate to **decreasing both** IgG1 and IgG2a serum antibody levels and is **not limited to** CII-specific antibodies.

12. A fundamental flaw with the Examiner's interpretation of the results of Myers is the Examiner's assumption that the change (increase) in CII-specific IgG2a and IgG1 antibody levels would be indicative of the same change in all other types of IgG2a and IgG1 antibodies. This is not the case. A scientist of ordinary skill in the art would expect that the arthritis-inducing CII vaccine, which was given to all experimental and control cohorts, would increase CII-specific IgG2a and IgG1 antibody levels, but not other kinds of antibodies as would be expected of all effective vaccines. Myers disclosed that the COX-2-/- experimental cohort failed to be efficiently immunized against the self CII antigen, resulting in the reduced level of CII-specific IgG2a and IgG1 antibody levels. Given the absence of any experimental data on non-CII-specific antibody levels, it is impossible to make, from Myers' work, any predictions on changes in non-CII-specific antibody levels (or global antibody levels) in the context of COX-2 or COX-1 gene deficiency, much less in the context of an arthritic versus a non-arthritic disease-state. Therefore, the Examiner's statement that treatment of RA would result in the

decrease of IgG1 and increase of IgG2a in patients has absolutely no basis in the context of the teaching of Myers.

13. The Examiner's statement that "[a] major factor in the inhibition of CIA in COX-2-/- mice is the inability of these mice to produce antibodies to CII" is inaccurate. *See* page 7 of the Office Action. More accurately, the experimental data disclosed by Myers indicates that a major factor in the inhibition of CIA in COX-2-/- mice is the inability of these mice to respond to the CII antigenic vaccine stimulus as evidenced by diminished anti-CII specific antibody production. Myers teaches that COX-2-/- mice had a significantly lower incidence of arthritis and exhibited two defects accounting for the impaired ability of the mice to develop CIA: "[a] reduced immune response to CII ***demonstrated by*** a markedly reduced antibody titer, and an 'inflammatory' defect ***reflected by*** the inability to passively transfer arthritis to COX-2-/- mice." *See* abstract of Myers at page 2687 (emphasis added).

The COX-2-/- mice of the Myers study were impaired in their development of arthritis, because the COX-2 deficiency prevented the mice from reacting to the trigger for developing arthritis, whereby the trigger is the immunization of the animal against CII such that they develop an auto-reactive (self-reactive) immune response against self-CII. Diminished anti-CII specific antibodies are merely the resulting effect of the inability of COX-2-/- mice to respond to the RA disease trigger of CII vaccination. The emphasized phrases "***demonstrated by***" and "***reflected by***" in the above quotation clearly reflect the "resulting effect" relationship of reduced anti-CII specific antibody production to the COX-2-/- immune/inflammatory defect. To clarify further, the direct cause of the non-responsiveness to the CIA-triggering vaccine in COX-2-/- mice is the inflammatory

defect resulting from COX-2 deficiency, and this inflammatory defect lies "upstream" of and controls anti-CII antibody production. For this reason, Myers does not support the notion that COX-2 inhibition could be a means of ***treating*** RA, because the COX-2 deficiency operated at the level of ***preventing the induction of the RA disease state*** (i.e., at the level of CII vaccination response). Therefore, Myers at most teaches that the reduction of an inflammatory response by the suppression of COX-2 activity could be an effective therapy to ***prevent*** RA. Given that COX-2-/- mice were inhibited at the level of RA acquisition, one of ordinary skill in the art could not predict the effects of COX-2 inhibition on the treatment of RA, much less the effects of COX-2 inhibition on serum antibody levels in an active RA disease state.

14. The general consensus in the scientific community at the time of the filing of this patent application was that treatment of RA results in **a decrease in the serum levels of IgG2a**. In contrast, the application claims a method for decreasing the serum level of IgG1 and **increasing the serum level of IgG2a** in a mammal in need thereof, said method comprising orally administering an extract of *Actinidia arguta* to said mammal, wherein said extract is provided in an amount sufficient to decrease the serum level of IgG1 and **increase the serum level of IgG2a** in said mammal.

This general consensus at the time of filing of this application is exemplified by RA. Yamaki, K. *et al. J. Pharm. Pharmacol. 55*: 1661-1666 (2003) (Exhibit H) ("Yamaki") and Mukherjee, P. *et al. Ann. Rheum. Dis. 62*: 707-714 (2003) (Exhibit I) ("Mukherjee"). Both Yamaki and Mukherjee disclosed that different RA therapies all resulted in a statistically significant reduction in IgG2a antibody levels in arthritic cohorts. Specifically, Yamaki states the following:

> The results showed that treatment with MTX was followed by decreases in OVA-specific IgG and proliferation of spleen cells to the antigen. ***The anti-rheumatic drug inhibited both anti-OVA IgG2a and IgG1 production***, although the inhibitory effect of MTX on the antigen-specific IgG2a production appeared to be greater than that on IgG1 production.

*See* Abstract of Yamaki at page 1661 (emphasis added).

Mukherjee describes similar findings to Yamaki in cohorts treated with a different RA therapy in the following statement:

> Results: Severity of CIA was significantly decreased in TNF-R treated animals compared with controls, 14-34 days after disease onset... Seven days after disease onset, ***TNF-R treated mice had lower levels of inflammatory Th1 driven IgG2a antibodies to CII than controls***... Conclusions: The overall influence of TNF-R gene therapy is that it ***inhibits the progression of CIA mainly by suppressing the inflammatory Th1 response*** rather than by stimulating a Th2 response. Therefore, periarticular TNF-R gene therapy may have excellent therapeutic potential in RA.

*See* Abstract of Mukherjee at page 707 (emphasis added).

Yamaki and Mukherjee, therefore, contradict the Examiner's claim, which was based on a misinterpretation of Myers, that "[t]reatment of arthritis would result in the decrease of IgG1 and ***increase of IgG2a*** in patients with arthritis." *See* Office Action at page 7.

15. It is my understanding that Yudoh *et al. Arthritis & Rheumatism 43(3)*:617-627 (2000) ("Yudoh"), which was cited against U.S. Appl. No. 10/646,145, is considered by the Examiner to suggest that treatment of rheumatoid arthritis (RA) would result in the decrease in Th2 serum cytokines and an increase in Th1 serum cytokines in patients. The Examiner's argument is incorrect and is based on a misinterpretation of Yudoh.

16. Yudoh states that "[i]n RA, reduced expression of the CD4+ T cell subset producing IL-10, but not IL-2 and IL-4, may be responsible for the dominance of Th1

over Th2 cells at sites of inflamed synovium and in the peripheral blood..." and "[d]ecreases in this type of CD4+ T cell subset may induce the down-regulation of T cell tolerance and exacerbate the inflammatory process in RA" *See* abstract of Yudoh at page 617. To paraphrase the above quotation, reduced expression of T regulatory type 1 (Tr1) cells, which are not to be confused with Th1 cells, is believed to be responsible for the increased Th1 inflammatory cells in arthritic joints and in the peripheral blood, and the reduced numbers of Tr1 cells in arthritic joints and in the peripheral blood may be responsible for the inflammatory disease associated with RA.

Based on the disclosure of Yudoh, a scientist would at most be motivated to treat RA by increasing the levels of Tr1 cells in arthritic joints or in the peripheral blood, which would result in a reduction of Th1 inflammatory cells in the arthritic joints or in the peripheral blood. The end result of such a therapeutic approach would be the **reduced expression of Th1 cytokines** in the arthritic joints or in the peripheral blood due to the reduction of Th1 inflammatory cells. In contrast, the present application claims a method for **simultaneously decreasing Th2 serum cytokines and increasing Th1 serum cytokines** in a mammal in need thereof, said method comprising orally administering an extract of Actinidia arguta to said mammal, wherein said extract is provided in an amount sufficient to **simultaneously decrease serum Th2 cytokines and increase Th1 cytokines** in said mammal.

17. Yudoh clearly indicates a link between RA disease and increased Th1 cytokines, which result from a reduced Tr1 cell population. Further support for the link between RA and increased serum Th1 cytokines was disclosed at the time of filing of the present application in Schulze-Koops, H. and Kalden, J. *Best Practice & Research*

*Clinical Rheumatology 15*: 677-691 (2001) (Exhibit J) ("Schulze-Koops") and Hermann, J. *et al. Springer Semin. Immunopathol. 20*: 275-288 (1998) (Exhibit K) ("Hermann"). Both Schulze-Koops and Hermann report that anti-arthritic therapies designed to shift the Th1/Th2 cytokine balance to a predominantly Th2 response could help reduce Th1-associated inflammatory cytokine production and inflammation at sites of RA disease.

In the following statement, Schulze-Koops clearly teaches both that RA is a disease driven by increased Th1 cellular activation and that shifting the Th1/Th2 balance towards increased Th2 cellular activation might be effective for treating RA:

> In RA, convincing arguments, both clinical and experimental, have been provided to suggest that autoimmune rheumatoid inflammation is also driven by activated Th1 effectors without sufficient Th2 generation to downregulate inflammation. Furthermore, recent data suggest that ***several treatment modalities currently employed in RA, exert their immunomodulatory effect at least in part by inhibiting Th1 cell activation*** and/or differentiation and by favouring Th2 differentiation, thereby shifting the Th1/Th2 balance towards the Th2 direction. Thus selective manipulation of Th cell differentiation to induce Th2 effectors might be a successful approach for interrupting ongoing and established Th1-driven chronic autoimmune diseases such as RA.

*See* Schulze-Koops at page 688 (emphasis added). Schulze-Koops further teaches that IL-4, which is a Th2 cytokine, is "[s]uitable for ameliorating signs and symptoms of chronic arthritis." *See* Schulze-Koops at page 681.

In accord with the teachings of Schulze-Koops, Hermann also teaches that increasing Th2-derived cytokines such as IL-4 could be useful in treating RA:

> This and other evidence suggests that CD4+ Th2-derived cytokines are not abundant in RA joints, and that CD4+ Th1 cells predominate in this site. It is possible that the lack of IL-4 producing CD4+ Th2 cells contributes to the pathogenesis of RA, and this has led to suggestions that IL-4 may be a useful therapeutic agent.

*See* Hermann at page 282.

Based on the teachings of Schulze-Koops, Hermann, and Yudoh (when properly interpreted as indicated above), the Examiner's statement that "treatment of rheumatoid arthritis (RA) would result in the decrease in Th2 serum cytokines and an increase in Th1 serum cytokines in patients" is unfounded on a factual basis. *See* Office Action at page 8. Given that RA treatments at the time of filing operate at the level of Th1 inhibition as evidenced by the teachings of Schulze-Koops, Hermann, and Yudoh, a scientist would at most be motivated to treat RA by increasing Th2 serum cytokines such as IL-4, which would be contrary to the claimed methods of the present application.

18. In conclusion, the arguments presented above in conjunction with the technical evidence presented in Exhibits B-K clearly demonstrate that the Examiner's assertions regarding the lack of novelty of the present invention are unfounded.

19. I further declare that the above statements made of my own knowledge are true and the above statements based on information and belief obtained from the references and documents discussed are believed to be true. Additionally, I declare that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Title 18 United States Code Section 1001, and that willful false statements may jeopardize the validity of this application or any patent issuing thereon.

20. I have read, I am familiar with, and I understand, the provisions of 37 C.F.R.§§ 11.18(b) and (c) relating to the effect of signature and certificate for correspondence filed in the U.S. Patent and Trademark Office.

Date: October 18, 2011

Sunyoung Kim, D. Phil.

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# Exhibit A

# CURRICULUM VITAE

Name : Sunyoung Kim
Position: Professor
Institution : School of Biological Sciences, Seoul National University

## Academic Degrees:

| | | | |
|---|---|---|---|
| 1978 | Microbiology | B.S. | Seoul National University |
| 1982 | Biochemical Engineering | M.S. | MIT |
| 1984 | Microbiology and Molecular Genetics | M.A. | Harvard University |
| 1986 | Molecular Genetics | D. Phil | University of Oxford |

## Professional Activities (Selected):

| | |
|---|---|
| 1987-1989 | Postdoctoral fellow<br>Whitehead Institute for Biomedical Research,<br>and Department of Biology, MIT (with Dr. David Baltimore) |
| 1990-1992 | Assistant Professor of Medicine (Virology)<br>Harvard University |
| 1992- Present | Professor<br>Seoul National University |
| 1998-present | Member of the Editorial Board<br>Journal of Gene Medicine (John Wiley & Sons Ltd.) |
| 2003-2008 | Member of the Editorial Board<br>Gene Therapy (Nature Publishing Group) |
| 2006-2008 | President<br>Korea Society of Gene Therapy |
| 2005-2009 | Chief Executive Officer, Founder<br>ViroMed Co. Ltd. (Korea) |

## Publications:

89. K. Koh, Y. Cha, **S. Kim**, and J. Kim (2009) tBHQ inhibits LPS-induced microglial activation via Nrf2-mediated suppression of p38 phosphorylation. *Biochem Biophys Res Commun.*,380(3):449-53.

88. Donghyun Kim, Seon Hee Kim, Eun-Jin Park, Chang-Yuil Kang, **Sunyoung Kim** (2009) Suppression of allergic diarrhea in murine OVA-induced food allergy model by PG102, a water-soluble extract prepared from Actinidia arguta. *International Archives of Allergy and Immunology*,150(2):164-71.

87. Kyungmi Koh, Karim Lee, Jinhyun Ahn, **Sunyoung Kim** (2009) Human cytomegalovirus infection downregulates the expression of glial fibrillary acidic protein in human glioblastoma cells, U373MG ; Identification of viral genes and protein domains involved. *Journal of General Virology*, 90(Pt 4):954-62.

86. Donghyun Kim, Seon Hee Kim, Eun-Jin Park, Chang-Yuil Kang, **Sunyoung Kim** (2009) Anti-allergic effects of PG102, a water-soluble extract prepared from Actinidia arguta, in a murine OVA-induced asthma model. *Clinical & Experimental Allergy*,39(2):280-9.

85. Youngtae Hong, Seung Shin Yu, Nam-Kyung Yoon, Sung June Kang, Jun-Tae Lee, Sujeoung Kim, Jong-Mook Kim, Karim Lee, Ji-Won Jang, **Sunyoung Kim** (2008.8) Development of an in vitro cell culture assay system for measuring the activation of a neighbouring gene by the retroviral vector. *The Journal of Gene Medicine*,10(8):847-54.

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# Exhibit B

1. Exploratory Data Analysis
1.3. EDA Techniques

# 1.3.5. Quantitative Techniques

*Confirmatory Statistics*

The techniques discussed in this section are classical statistical methods as opposed to EDA techniques. EDA and classical techniques are not mutually exclusive and can be used in a complamentary fashion. For example, the analysis can start with some simple graphical techniques such as the 4-plot followed by the classical confirmatory methods discussed herein to provide more rigorous statments about the conclusions. If the classical methods yield different conclusions than the graphical analysis, then some effort should be invested to explain why. Often this is an indication that some of the assumptions of the classical techniques are violated.

Many of the quantitative techniques fall into two broad categories:

1. Interval estimation
2. Hypothesis tests

*Interval Estimates*

It is common in statistics to estimate a parameter from a sample of data. The value of the parameter using all of the possible data, not just the sample data, is called the population parameter or true value of the parameter. An estimate of the true parameter value is made using the sample data. This is called a point estimate or a sample estimate.

For example, the most commonly used measure of location is the mean. The population, or true, mean is the sum of all the members of the given population divided by the number of members in the population. As it is typically impractical to measure every member of the population, a random sample is drawn from the population. The sample mean is calculated by summing the values in the sample and dividing by the number of values in the sample. This sample mean is then used as the point estimate of the population mean.

Interval estimates expand on point estimates by incorporating the uncertainty of the point estimate. In the example for the mean above, different samples from the same population will generate different values for the sample mean. An interval estimate quantifies this uncertainty in the sample estimate by computing lower and upper

values of an interval which will, with a given level of confidence (i.e., probability), contain the population parameter.

*Hypothesis Tests*

Hypothesis tests also address the uncertainty of the sample estimate. However, instead of providing an interval, a hypothesis test attempts to refute a specific claim about a population parameter based on the sample data. For example, the hypothesis might be one of the following:

- the population mean is equal to 10
- the population standard deviation is equal to 5
- the means from two populations are equal
- the standard deviations from 5 populations are equal

To reject a hypothesis is to conclude that it is false. However, to accept a hypothesis does not mean that it is true, only that we do not have evidence to believe otherwise. Thus hypothesis tests are usually stated in terms of both a condition that is doubted (null hypothesis) and a condition that is believed (alternative hypothesis).

A common format for a hypothesis test is:

| | |
|---|---|
| $H_0$: | A statement of the null hypothesis, e.g., two population means are equal. |
| $H_a$: | A statement of the alternative hypothesis, e.g., two population means are not equal. |
| Test Statistic: | The test statistic is based on the specific hypothesis test. |
| Significance Level: | The significance level, $\alpha$, defines the sensitivity of the test. A value of $\alpha = 0.05$ means that we inadvertently reject the null hypothesis 5% of the time when it is in fact true. This is also called the type I error. The choice of $\alpha$ is somewhat arbitrary, although in practice values of 0.1, 0.05, and 0.01 are commonly used.<br><br>The probability of rejecting the null hypothesis when it is in fact false is called the power of the test and is denoted by $1 - \beta$. Its complement, the probability of accepting the null hypothesis when the alternative hypothesis is, in fact, true (type II error), is called $\beta$ and can only be computed for a specific alternative hypothesis. |

Critical Region: The critical region encompasses those values of the test statistic that lead to a rejection of the null hypothesis. Based on the distribution of the test statistic and the significance level, a cut-off value for the test statistic is computed. Values either above or below or both (depending on the direction of the test) this cut-off define the critical region.

*Practical Versus Statistical Significance*

It is important to distinguish between statistical significance and practical significance. Statistical significance simply means that we reject the null hypothesis. The ability of the test to detect differences that lead to rejection of the null hypothesis depends on the sample size. For example, for a particularly large sample, the test may reject the null hypothesis that two process means are equivalent. However, in practice the difference between the two means may be relatively small to the point of having no real engineering significance. Similarly, if the sample size is small, a difference that is large in engineering terms may not lead to rejection of the null hypothesis. The analyst should not just blindly apply the tests, but should combine engineering judgement with statistical analysis.

*Bootstrap Uncertainty Estimates*

In some cases, it is possible to mathematically derive appropriate uncertainty intervals. This is particularly true for intervals based on the assumption of a normal distribution. However, there are many cases in which it is not possible to mathematically derive the uncertainty. In these cases, the bootstrap provides a method for empirically determining an appropriate interval.

*Table of Contents*

Some of the more common classical quantitative techniques are listed below. This list of quantitative techniques is by no means meant to be exhaustive. Additional discussions of classical statistical techniques are contained in the product comparisons chapter.

- Location
    1. Measures of Location
    2. Confidence Limits for the Mean and One Sample t-Test
    3. Two Sample t-Test for Equal Means
    4. One Factor Analysis of Variance
    5. Multi-Factor Analysis of Variance
- Scale (or variability or spread)
    1. Measures of Scale
    2. Bartlett's Test

3. Chi-Square Test
4. F-Test
5. Levene Test

- Skewness and Kurtosis
  1. Measures of Skewness and Kurtosis
- Randomness
  1. Autocorrelation
  2. Runs Test
- Distributional Measures
  1. Anderson-Darling Test
  2. Chi-Square Goodness-of-Fit Test
  3. Kolmogorov-Smirnov Test
- Outliers
  1. Grubbs Test
- 2-Level Factorial Designs
  1. Yates Analysis

1. Exploratory Data Analysis
1.3. EDA Techniques
1.3.5. Quantitative Techniques

# 1.3.5.3. Two-Sample *t*-Test for Equal Means

*Purpose: Test if two population means are equal*

The two-sample *t*-test (Snedecor and Cochran, 1989) is used to determine if two population means are equal. A common application of this is to test if a new process or treatment is superior to a current process or treatment.

There are several variations on this test.

1. The data may either be paired or not paired. By paired, we mean that there is a one-to-one correspondence between the values in the two samples. That is, if $X_1, X_2, ..., X_n$ and $Y_1, Y_2, ..., Y_n$ are the two samples, then $X_i$ corresponds to $Y_i$. For paired samples, the difference $X_i$ - $Y_i$ is usually calculated. For unpaired samples, the sample sizes for the two samples may or may not be equal. The formulas for paired data are somewhat simpler than the formulas for unpaired data.
2. The variances of the two samples may be assumed to be equal or unequal. Equal variances yields somewhat simpler formulas, although with computers this is no longer a significant issue.
3. In some applications, you may want to adopt a new process or treatment only if it exceeds the current treatment by some threshold. In this case, we can state the null hypothesis in the form that the difference between the two populations means is equal to some constant ($\mu_1 - \mu_2 = d_0$) where the constant is the desired threshold.

*Definition*

The two sample t test for unpaired data is defined as:

$H_0$: $\mu_1 = \mu_2$

$H_a$: $\mu_1 \neq \mu_2$

Test Statistic:

$$T = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{s_1^2/N_1 + s_2^2/N_2}}$$

where $N_1$ and $N_2$ are the sample sizes, $\bar{Y}_1$ and $\bar{Y}_2$ are the sample means, and $s_1^2$ and $s_2^2$ are the sample variances.

If equal variances are assumed, then the formula reduces to:

$$T = \frac{\bar{Y}_1 - \bar{Y}_2}{s_p\sqrt{1/N_1 + 1/N_2}}$$

where

$$s_p^2 = \frac{(N_1 - 1)s_1^2 + (N_2 - 1)s_2^2}{N_1 + N_2 - 2}$$

Significance Level: $\alpha$.

Critical Region: Reject the null hypothesis that the two means are equal if

$$T < -t_{(\alpha/2,\upsilon)}$$

or

$$T > t_{(\alpha/2,\upsilon)}$$

where $t_{(\alpha/2,\upsilon)}$ is the critical value of the t distribution with $\upsilon$ degrees of freedom where

$$\upsilon = \frac{(s_1^2/N_1 + s_2^2/N_2)^2}{(s_1^2/N_1)^2/(N_1 - 1) + (s_2^2/N_2)^2/(N_2 - 1)}$$

If equal variances are assumed, then

$$\upsilon = N_1 + N_2 - 2$$

*Sample Output*

Dataplot generated the following output for the t test from the AUTO83B.DAT data set:

```
                          T TEST
                       (2-SAMPLE)
 NULL HYPOTHESIS UNDER TEST--POPULATION MEANS MU1 = MU2

 SAMPLE 1:
    NUMBER OF OBSERVATIONS            =       249
    MEAN                              =    20.14458
    STANDARD DEVIATION                =    6.414700
    STANDARD DEVIATION OF MEAN        =   0.4065151
```

```
SAMPLE 2:
   NUMBER OF OBSERVATIONS       =        79
   MEAN                         =     30.48101
   STANDARD DEVIATION           =     6.107710
   STANDARD DEVIATION OF MEAN   =    0.6871710

IF     ASSUME SIGMA1 = SIGMA2:
   POOLED STANDARD DEVIATION    =     6.342600
   DIFFERENCE (DEL) IN MEANS    =    -10.33643
   STANDARD DEVIATION OF DEL    =    0.8190135
   T TEST STATISTIC VALUE       =    -12.62059
   DEGREES OF FREEDOM           =     326.0000
   T TEST STATISTIC CDF VALUE   =     0.000000

IF NOT ASSUME SIGMA1 = SIGMA2:
   STANDARD DEVIATION SAMPLE 1  =     6.414700
   STANDARD DEVIATION SAMPLE 2  =     6.107710
   BARTLETT CDF VALUE           =     0.402799
   DIFFERENCE (DEL) IN MEANS    =    -10.33643
   STANDARD DEVIATION OF DEL    =    0.7984100
   T TEST STATISTIC VALUE       =    -12.94627
   EQUIVALENT DEG. OF FREEDOM   =     136.8750
   T TEST STATISTIC CDF VALUE   =     0.000000
```

| ALTERNATIVE-HYPOTHESIS | ALTERNATIVE-HYPOTHESIS ACCEPTANCE INTERVAL | ALTERNATIVE-HYPOTHESIS CONCLUSION |
|---|---|---|
| MU1 <> MU2 | (0,0.025) (0.975,1) | ACCEPT |
| MU1 < MU2 | (0,0.05) | ACCEPT |
| MU1 > MU2 | (0.95,1) | REJECT |

*Interpretation of Sample Output*

We are testing the hypothesis that the population mean is equal for the two samples. The output is divided into five sections.

1. The first section prints the sample statistics for sample one used in the computation of the *t*-test.
2. The second section prints the sample statistics for sample two used in the computation of the *t*-test.
3. The third section prints the pooled standard deviation, the difference in the means, the *t*-test statistic value, the degrees of freedom, and the cumulative distribution function (cdf) value of the *t*-test statistic under the assumption that the standard deviations are equal. The *t*-test statistic cdf value is an alternative way of expressing the critical value. This cdf value is compared to the acceptance intervals printed in section five. For an upper one-tailed test, the acceptance interval is $(0,1-\alpha)$, the acceptance interval for a two-tailed test is $(\alpha/2, 1-\alpha/2)$, and the acceptance interval for a lower

one-tailed test is ($\alpha$,1).

4. The fourth section prints the pooled standard deviation, the difference in the means, the *t*-test statistic value, the degrees of freedom, and the cumulative distribution function (cdf) value of the *t*-test statistic under the assumption that the standard deviations are not equal. The *t*-test statistic cdf value is an alternative way of expressing the critical value. cdf value is compared to the acceptance intervals printed in section five. For an upper one-tailed test, the alternative hypothesis acceptance interval is (1 - $\alpha$,1), the alternative hypothesis acceptance interval for a lower one-tailed test is (0,$\alpha$), and the alternative hypothesis acceptance interval for a two-tailed test is (1 - $\alpha$/2,1) or (0,$\alpha$/2). Note that accepting the alternative hypothesis is equivalent to rejecting the null hypothesis.
5. The fifth section prints the conclusions for a 95% test under the assumption that the standard deviations are not equal since a 95% test is the most common case. Results are given in terms of the alternative hypothesis for the two-tailed test and for the one-tailed test in both directions. The alternative hypothesis acceptance interval column is stated in terms of the cdf value printed in section four. The last column specifies whether the alternative hypothesis is accepted or rejected. For a different significance level, the appropriate conclusion can be drawn from the *t*-test statistic cdf value printed in section four. For example, for a significance level of 0.10, the corresponding alternative hypothesis acceptance intervals are (0,0.05) and (0.95,1), (0, 0.10), and (0.90,1).

Output from other statistical software may look somewhat different from the above output.

*Questions*

Two-sample *t*-tests can be used to answer the following questions:

1. Is process 1 equivalent to process 2?
2. Is the new process better than the current process?
3. Is the new process better than the current process by at least some pre-determined threshold amount?

*Related Techniques*

Confidence Limits for the Mean
Analysis of Variance

*Case Study*

Ceramic strength data.

*Software*

Two-sample *t*-tests are available in just about all general purpose statistical software programs, including Dataplot.

# Exhibit C

Performing your original search, ***effects of a nitric oxide donor on and correlation of changes in cyclic nucleotide levels with*** , in PubMed will retrieve *8* records.

Neurosurgery. 2003 Mar;52(3):661-7; discussion 666-7.

# Effects of a nitric oxide donor on and correlation of changes in cyclic nucleotide levels with experimental vasospasm.

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## Abstract

**OBJECTIVE:** Vasospasm after subarachnoid hemorrhage (SAH) may result from hemoglobin-mediated removal of nitric oxide (NO) from the arterial wall. We tested the ability of the long-acting, water-soluble, NO donor (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-1,2-diolate (DETA/NO), delivered via continuous intracisternal infusion, to prevent vasospasm in a nonhuman primate model of SAH.

**METHODS:** First, vasorelaxation in response to DETA/NO was characterized in vitro by using monkey basilar artery rings under isometric tension. Next, monkeys were randomized to undergo angiography, unilateral SAH, and no treatment (SAH only, n = 4) or treatment with DETA/NO (1 mmol/L, 12 ml/d, n = 4) or decomposed DETA/NO (at the same dose, n = 4). Vasospasm was assessed by angiography, which was performed on Day 0 and Day 7. Levels of cyclic adenosine monophosphate and cyclic guanosine monophosphate (cGMP) were measured in cerebral arteries on Day 7.

**RESULTS:** DETA/NO produced significant relaxation of monkey arteries in vitro, which reached a maximum at concentrations of 10(-5) mol/L. In monkeys, angiography demonstrated significant vasospasm of the right intradural cerebral arteries in all three groups, with no significant difference in vasospasm among the groups ($P > 0.05$, analysis of variance). The ratios of cGMP or cyclic adenosine monophosphate levels in the right and left middle cerebral arteries were not different among the groups ($P > 0.05$, analysis of variance). There was no significant correlation between arterial cGMP contents and the severity of vasospasm.

**CONCLUSION:** DETA/NO did not prevent vasospasm. There was no correlation between the severity of vasospasm and cyclic adenosine monophosphate and cGMP levels in the cerebral arteries. These results suggest that events downstream of cyclic nucleotides may be abnormal during vasospasm.

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Publication Types, MeSH Terms, Substances

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# Exhibit D1

Behavioral/Systems/Cognitive

# Disparity-Based Coding of Three-Dimensional Surface Orientation by Macaque Middle Temporal Neurons

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Gradients of binocular disparity across the visual field provide a potent cue to the three-dimensional (3-D) orientation of surfaces in a scene. Neurons selective for 3-D surface orientation defined by disparity gradients have recently been described in parietal cortex, but little is known about where and how this selectivity arises within the visual pathways. Because the middle temporal area (MT) has previously been implicated in depth perception, we tested whether MT neurons could signal the 3-D orientation (as parameterized by tilt and slant) of planar surfaces that were depicted by random-dot stereograms containing a linear gradient of horizontal disparities. We find that many MT neurons are tuned for 3-D surface orientation, and that tilt and slant generally have independent effects on MT responses. This separable coding of tilt and slant is reminiscent of the joint coding of variables in other areas (e.g., orientation and spatial frequency in V1). We show that tilt tuning remains unchanged when all coherent motion is removed from the visual stimuli, indicating that tilt selectivity is not a byproduct of 3-D velocity coding. Moreover, tilt tuning is typically insensitive to changes in the mean disparity (depth) of gradient stimuli, indicating that tilt tuning cannot be explained by conventional tuning for frontoparallel disparities. Finally, we explore the receptive field mechanisms underlying selectivity for 3-D surface orientation, and we show that tilt tuning arises through heterogeneous disparity tuning within the receptive fields of MT neurons. Our findings show that MT neurons carry high-level signals about 3-D surface structure, in addition to coding retinal image velocities.

*Key words:* visual cortex; extrastriate; stereopsis; binocular disparity; surface; tilt; slant

## Introduction

A typical visual environment contains a variety of surfaces at different three-dimensional (3-D) orientations relative to one's line of sight. For planar surfaces, the 3-D orientation can be described in terms of "tilt" and "slant" (see Fig. 1*A*). Accurate information about 3-D surface orientation is important for visual navigation and object manipulation, as well as for object recognition itself. Many visual cues can be used to judge the tilt and slant of surfaces, including texture gradients, velocity gradients, shading, perspective, and binocular disparity gradients (Sedgwick, 1986; Howard and Rogers, 2002). Disparity gradients are quantitatively related to 3-D surface orientation, knowing only the positions of the two eyes, whereas interpretation of other cues requires additional knowledge about object structure, observer motion, lighting, etc. Thus, disparity gradients provide robust information about 3-D surface orientation.

Recent physiological studies have described neurons in parietal and temporal cortex that are sensitive to 3-D structure defined by disparity gradients. Taira et al. (2000) and Tsutsui et al. (2002) have reported that neurons in the caudal intraparietal (CIP) area signal surface tilt defined by disparity gradients. Meanwhile, Janssen et al. (1999, 2000, 2001) have shown that inferotemporal neurons signal 3-D shape via disparity gradients. Although these studies establish the presence of disparity gradient signals at the upper levels of the dorsal and ventral processing streams, the origins and mechanisms of gradient selectivity in visual cortex remain unknown.

Computational and psychophysical studies (Gibson, 1950; Marr, 1982; Nakayama, 1996) suggest that 3-D surface structure should be computed early in the visual pathways. It seems unlikely that disparity gradients could be effectively coded in areas V1 or V2 because of the small size of receptive fields in these areas. We reasoned that the middle temporal area (MT) might participate in gradient computations because the receptive fields of MT neurons are several-fold larger than those of their primary inputs from V1/V2 (Albright and Desimone, 1987; Maunsell and Van Essen, 1987). In addition, recent studies have shown that MT contains strong disparity signals (Maunsell and Van Essen, 1983a; DeAngelis and Uka, 2003), that disparity-selective MT neurons are organized topographically (DeAngelis and Newsome, 1999), and that electrical stimulation of MT influences depth perception (DeAngelis et al., 1998). We therefore tested whether MT neurons signal the tilt and slant of 3-D surfaces defined solely by disparity gradients.

Two critical factors to control in these experiments are vergence eye movements and stimulus centering on the receptive field. Systematic changes in vergence angle with tilt or slant could give rise to artifactual tuning for surface orientation. Similarly,

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improper centering of the gradient stimulus on the receptive field can give a false impression of tilt selectivity unless tilt tuning is shown to be invariant to changes in the mean disparity of the gradient stimulus. These factors have not been rigorously controlled in previous studies (Janssen et al., 1999; Taira et al., 2000), whereas our experiments and analyses were designed specifically to account for them.

We show that many MT neurons exhibit robust tuning for the tilt and slant of disparity-defined surfaces. Our results complement previous studies of the responses of MT neurons to speed gradients (Treue and Andersen, 1996; Xiao et al., 1997) and suggest that MT neurons use multiple cues for computing 3-D surface orientation. These findings offer additional evidence that area MT plays important roles in 3-D vision.

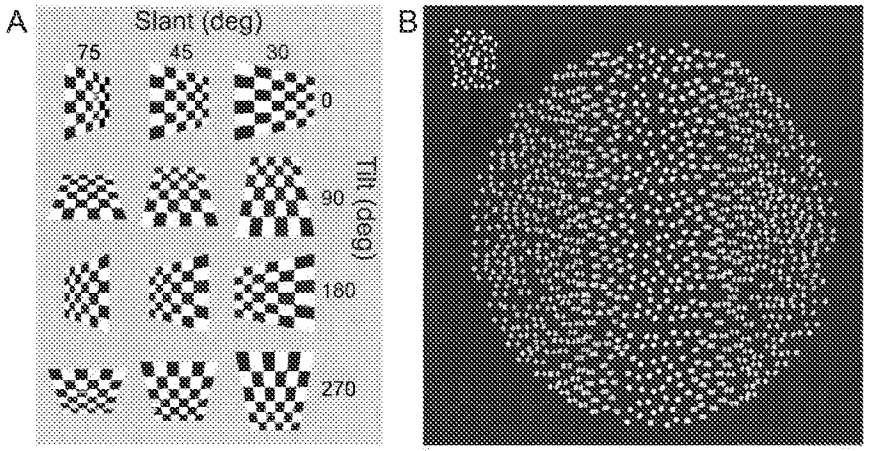


**Figure 1.** Schematic illustration of the 3-D orientation of planar surfaces, parameterized by tilt and slant. *A*, Tilt refers to the axis around which the plane is rotated away from frontoparallel, and slant defines the amount by which the plane is rotated (Gibson, 1950; Stevens, 1983). Zero slant corresponds to a frontoparallel surface for which the tilt is undefined. In this illustration, tilt and slant are defined by perspective and texture gradient cues. In our experiments, surface orientation was defined solely by the direction and magnitude of a linear gradient of horizontal disparity. *B*, An example of a 0 degree tilt stimulus depicted using a red–green anaglyph. Dots in our experiments were all red, and stereoscopic presentation was accomplished using ferroelectric liquid crystal shutters synchronized to the monitor refresh.

## Materials and Methods

Two male rhesus monkeys (*Macaca mulatta*), weighing between 5 and 7 kg, performed a standard fixation task during extracellular recording experiments. A detailed description of our methods has recently appeared (DeAngelis and Uka, 2003); here, we briefly review these procedures, focusing on those aspects most relevant to the present study. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Washington University and conformed to National Institutes of Health guidelines.

*Visual stimuli.* Stereoscopic visual stimuli were presented using frame alternation (at 100 Hz) on a 22 inch flat-face monitor that subtended $40 \times 30°$ at the viewing distance of 57 cm. Random-dot stereograms were generated by an OpenGL accelerator board (3 Dlabs Oxygen GVX1) and were viewed by the monkey through ferroelectric liquid crystal shutters that were synchronized to the monitor refresh. Stereo crosstalk was ~3%. Some of the later experiments (including monocular controls) (see Fig. 5) were performed using a stereoscopic projector (Christie Digital Mirage 2000; image subtense: $56 \times 46°$) that had no measurable stereo crosstalk; similar results were obtained using both display devices.

Stereograms consisted of red dots (~0.1° diameter) presented on a black background. Dot density was generally 64 dots per square degree per second, and dots were presented within a circular aperture. Precise disparities and smooth motion were achieved by plotting dots with subpixel resolution using hardware anti-aliasing under OpenGL. Except where noted in the text, dots moved coherently at the preferred direction and speed of each MT neuron and wrapped around when they reached the edge of the aperture.

In these experiments, 3-D surface orientation was varied by applying linear gradients of horizontal disparities to the random-dot stereograms. It is important to note that the disparity gradient was the only useful cue to surface orientation in these stimuli: there were no corresponding speed or texture gradients in the stimulus as would typically occur for a real slanted surface in a natural scene. Note, however, that application of a disparity gradient does produce very subtle variations in dot density along the axis of the gradient. We thus performed monocular controls (described below) (see Fig. 5) to exclude the possibility that these subtle monocular density cues account for tilt tuning.

*Task and data collection.* Monkeys were required to maintain their conjugate eye position within a 1.5° diameter fixation window that was centered on the fixation point. Fixation began 300 msec before presentation of the random-dot stereogram and had to be maintained throughout the 1.5 sec stimulus presentation to receive a liquid reward. Only data from successfully completed trials were analyzed. Movements of both eyes were measured in all experiments using eye coils that were sutured to the sclera; eye position signals were stored to disk at 250 Hz.

Tungsten microelectrodes were introduced into the cortex through a transdural guide tube, and area MT was recognized based on the following criteria: the pattern of gray and white matter transitions along electrode penetrations, the response properties of single units and multiunit clusters (direction, speed, and disparity tuning), retinal topography, the relationship between receptive field size and eccentricity, and the subsequent entry into gray matter with response properties typical of the medial superior temporal area. All data included in this study were taken from portions of electrode penetrations that were confidently assigned to area MT. Raw neural signals were amplified and bandpass filtered (500–5000 Hz) using conventional electronic equipment. Action potentials of single MT units were isolated using a dual voltage–time window discriminator (Bak Electronics) and time-stamped with 1 msec resolution.

*Experimental protocol.* The receptive field (RF) of each isolated MT neuron was initially explored using a mapping program to carefully estimate the RF location and size, preferred velocity, and preferred disparity. We subsequently performed the following series of quantitative tests on each MT neuron (each condition below represents a separate block of trials). (1) A direction-tuning curve was obtained by presenting moving random-dot patterns at eight directions of motion, 45° apart. (2) A speed-tuning curve was obtained by presenting random-dot patterns at speeds of 0, 0.5, 1, 2, 4, 8, 16, and 32°/sec, with direction fixed to the optimal value. (3) Horizontal disparity tuning was measured by presenting moving random dots at nine disparities typically ranging from −1.6 to 1.6° in steps of 0.4°. These parameters were adjusted as necessary based on the initial RF exploration. (4) The receptive field was mapped quantitatively by presenting small (<0.25 × RF size) rectangular patches of moving dots at 16 locations on a $4 \times 4$ grid that covered the receptive field. A two-dimensional Gaussian was fit to this RF map to determine the center location of the receptive field. (5) A size-tuning curve was obtained by presenting moving random dots in circular apertures having sizes of 0, 1, 2, 4, 8, 16, and 32°. Results of this test were used to quantify the extent (percent) of surround inhibition exhibited by each neuron (DeAngelis and Uka, 2003). (6) Tilt tuning was assessed by presenting stereograms containing a linear gradient of horizontal disparities across the circular aperture (Fig. 1*B*). The stimuli depicted surfaces at eight tilt angles, 45 degrees apart (see Fig. 1*A* for convention). Each tilt angle was presented at three to five different mean disparities that typically flanked the peak in the disparity tuning curve of the neuron (see Fig. 3*A*,*B*,*D*). The magnitude of the disparity gradient was 0.15°/° for most experi-

ments, corresponding to a surface slanted ~67 degrees away from frontoparallel. We chose a steep slant to maximize our chances of observing tilt tuning in this test (similar to Xiao et al., 1997).

Stimulus size for the tilt-tuning measurements was chosen based on the results of the receptive-field mapping and size-tuning experiments. For neurons that did not show any surround inhibition in the size-tuning test, stimulus size was chosen to encompass the entire classical receptive field (including the weakest flanks) as mapped using the 4 × 4 grid described above. For neurons with clear surround inhibition, stimulus size was chosen to be two or three times larger than the stimulus that elicited a maximal response, so that the stimulus encompassed a large portion of the nonclassical inhibitory surround. In some cases of exceptionally strong surround inhibition, however, a stimulus two or three times the optimal size elicited little or no response from the neuron. In these instances, stimulus size was reduced until the neuron gave an approximately half-maximal response. Because we found no overall correlation between tilt selectivity and the strength of surround inhibition (see Fig. 10), our population analyses were done by combining data across neurons regardless of the presence of surround inhibition.

Because the relationship between disparity and depth is nonlinear, our linear disparity gradients depict surfaces that are not exactly planar (although this departure is generally not evident to human observers). Slant is not constant across space when the stimulus is large, and slant also varies a bit with the mean disparity of the gradient stimulus. However, given that tilt tuning was observed across a variety of stimulus sizes and that tilt tuning is generally invariant to changes in both mean disparity and slant, the subtle deviations from planarity in our stimuli cannot explain our results.

The above set of tests was performed on all 97 neurons included in the present study. For some neurons, we also performed one or more of the following additional tests. (1) The interaction between tilt and slant was examined for 29 neurons by presenting eight tilts at each of five to seven different slants chosen from the following set of gradient magnitudes: 0.001, 0.002, 0.01, 0.02, 0.05, 0.1, 0.15, 0.2, or 0.25°/°. These correspond to slants of 0.75, 1.5, 7, 15, 35, 54, 67, 73, and 76 degrees, measured at the center of a stimulus with zero mean disparity. (2) The effect of removing coherent motion from the stimulus was assessed by testing 10 neurons with stereograms in which dots were either stationary or randomly replotted every fourth video frame (0% motion coherence). (3) For 15 neurons, monocular tilt-tuning controls were obtained by turning off the dots presented to either the left or right eye while the image to the other eye was presented intact. (4) For some neurons with strong tilt selectivity, we probed the 3-D substructure of the receptive field by presenting pairs of circular patches of random dots. One member of the pair was always centered on the classical RF, and the other member of the pair was chosen from six locations surrounding the center stimulus (see Fig. 8*C*). The disparity of the center patch was held fixed at the optimal value, whereas the disparity of the surrounding patches varied from −2 to 2° in steps of 0.5°. This allowed us to measure a disparity-tuning curve for each of the six surrounding locations (see Fig. 8*D*). For neurons without surround inhibition, the entire array of seven patches was presented within the classical RF, such that the center patch was approximately one-third the size of the RF. When surround inhibition was present, the center patch was set to the optimal size (from the size-tuning curve), and the six surrounding patches extended into the inhibitory surround. Thus, our experiments probed for heterogeneous disparity tuning within either the classical RF or the nonclassical inhibitory surround (when present). In most cases, the center patch had the same dimensions as each of the six surrounding patches, but sometimes the size of the center patch was reduced to enhance the response modulations produced by varying the disparities of the six surrounding patches.

*Data analysis.* The response to each stimulus presentation was quantified as the average firing rate over the 1.5 sec stimulus period. Each different stimulus was typically presented five times in blocks of randomly interleaved trials. Tuning curves were constructed by plotting the mean ± SE of the response across repetitions of each different stimulus.

Each tilt-tuning curve was fit with a modified sinusoid having the following form:

$$R(\theta) = A \times G(\sin(f\theta + \psi)) + R_o, \quad (1)$$

where

$$G(x) = \frac{e^{nx} - 1}{n}, \quad (2)$$

$\theta$ denotes the tilt angle, and $A$, $f$, $\psi$, $R_o$, and $n$ are free parameters. $G(x)$ is an exponential function that can distort the sinusoid such that the peak is taller than the trough or vice versa. We found that this distortion of the sinusoid was necessary to fit the tilt-tuning curves of some MT neurons (see Figs. 8*E*, 9*A*). The best fit of this function to the data was achieved by minimizing the sum squared error between the responses of the neuron and the values of the function, using the constrained minimization tool, "lsqcurvefit", in Matlab (Mathworks). To homogenize the variance of the neural responses across different stimulus values, we minimized the difference between the square root of the neural responses and the square root of the function (Prince et al., 2002). Curve fits were generally quite good, accounting for 85% (median across all neurons) of the variance in MT responses. Additional details about our fitting procedures are described elsewhere (DeAngelis and Uka, 2003).

The frequency, $f$, of the modified sinusoid was constrained to lie within a range from 0.4 to 1.6. Although most of the fitted values of $f$ were very close to unity, the fits for a minority of neurons were significantly improved when the frequency was allowed to differ from unity. This could present a problem if we were using the phase parameter, $\psi$, of the fits to characterize the stimulus preference. However, tilt preferences were always computed by finding the actual peak of the modified sinusoid, such that there is no difficulty associated with frequencies that depart somewhat from unity.

To test if tilt tuning was sensitive to changes in slant or mean disparity (depth), we analyzed the data using two different models. In the first model, we fit the tilt-tuning curve for each different slant or mean disparity with an independent sinusoid given by Equation 1. We then computed the total sum-squared error of the independent fits. In the second model, we fit all tilt-tuning curves simultaneously while forcing the phase ($\psi$) and frequency ($f$) parameters of the sinusoids to be shared (constrained fits). The remaining parameters had independent values for each curve. This second model constrains the fitted curves to have identical peak and trough locations (i.e., a constant preferred tilt) while allowing them to have different amplitudes and mean responses. We then compared the total error of the constrained fits to that of the independent fits using a sequential $F$ test (Draper and Smith, 1966), with a significance criterion of $p < 0.05$. If the difference between models is insignificant ($p > 0.05$), we can conclude that the tilt preference is invariant to changes in slant or mean disparity.

## Results

We recorded from 203 neurons in two alert rhesus monkeys that performed a standard fixation task. There were no intentional selection criteria for sampling neurons, so the sample should be unbiased. We isolated 97/203 neurons long enough to obtain a complete set of data, which required the monkey to execute at least 486 correct trials (see Materials and Methods).

Figure 2 shows data for an exemplar neuron. This MT unit preferred far (uncrossed) disparities (Fig. 2*A*) and exhibited powerful surround inhibition when the diameter of the stimulus aperture was increased beyond a few degrees of visual angle (Fig. 2*B*). After mapping the RF quantitatively (Fig. 2*C*), we centered a 6° stimulus aperture (dashed circle) over the receptive field. This size was chosen to cover most of the excitatory RF without eliciting too much surround inhibition. In this aperture, we presented stereograms that simulated planar surfaces at eight tilt angles (45 degrees apart) relative to the line of sight; the simulated slant angle was 70 degrees. Figure 2*D* shows neuronal response

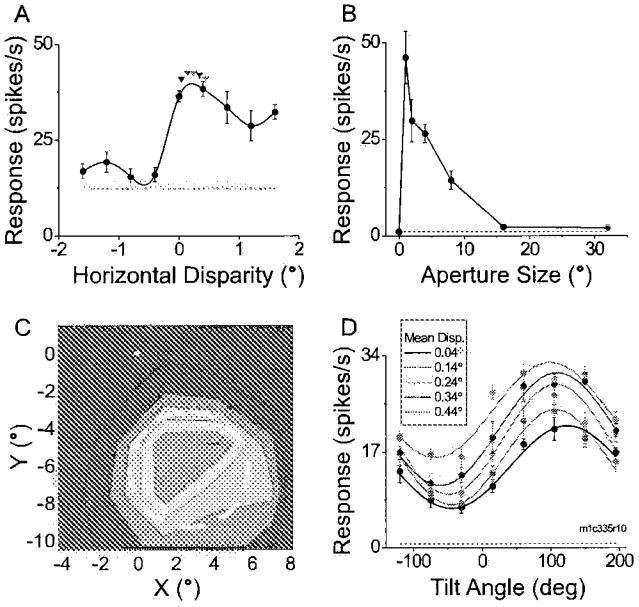


**Figure 2.** A dataset for an example MT neuron that exhibits tilt selectivity. *A*, A conventional disparity-tuning curve measured using random-dot stereograms (i.e., slant was zero, and different uniform horizontal disparities were applied). Mean responses ± SE are shown for each different stimulus disparity, along with a spline fit. Colored arrowheads indicate the five mean disparities used for the disparity gradients in *D*. *B*, A size-tuning (area summation) curve. A frontoparallel (zero slant) surface was presented at the preferred disparity, and the diameter of the stimulus aperture varied. The response of this neuron was abolished at large sizes, indicating the presence of powerful (96%) surround inhibition. *C*, A quantitative receptive-field map was measured by presenting small (1.3 × 1.3°) patches of dots at 16 spatial locations on a 4 × 4 grid. Response strength is color-coded, from low (dark blue) to high (red); peak response was 45 spikes/sec. The dashed white circle shows the location and size of the stimulus aperture in which disparity gradient stimuli were presented. *D*, Tilt-tuning curves at five different mean disparities (color-coded). Smooth curves indicate the best fits of the modified sinusoid (Eqs. 1, 2). Stimulus parameters were as follows: direction of motion, 105° (convention: rightward, 0°; upward, 90°); speed of motion, 17°/sec; aperture diameter, 6°; eccentricity, 6.8°; and gradient magnitude, 0.2°/°.

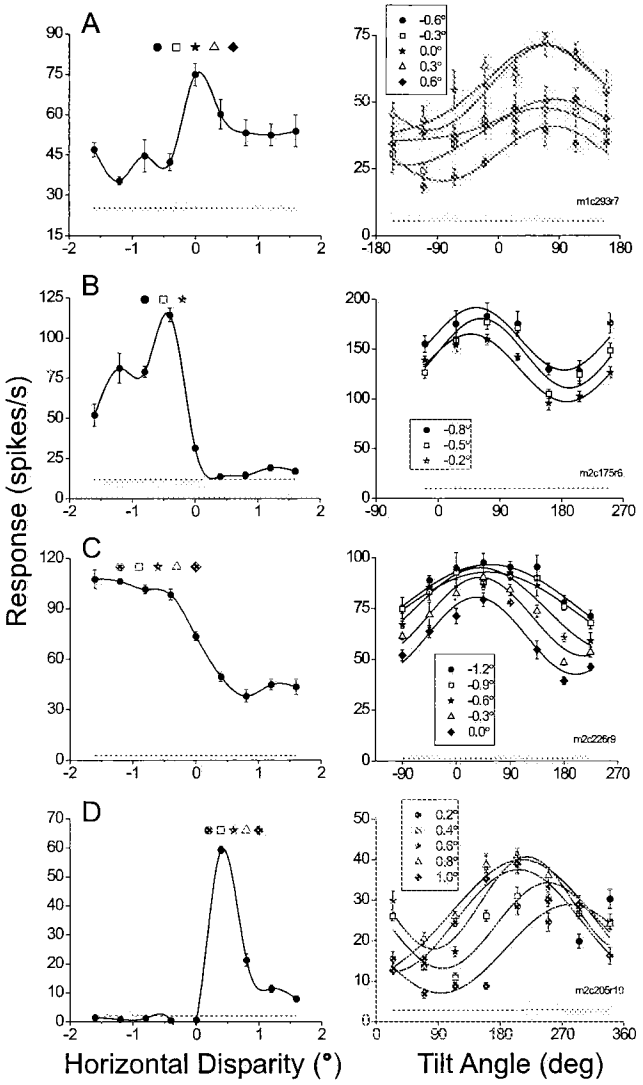


**Figure 3.** Horizontal disparity-tuning curves (left) and tilt-tuning curves (right) for four additional MT neurons. The format is similar to that of Figure 2, *A* and *D*, except that different mean disparities of the gradient stimulus are denoted here by different symbol types. Stimulus parameters were as follows: *A*, direction of motion, 70°; speed of motion, 1°/sec; aperture diameter, 4°; eccentricity, 5.5°; surround inhibition, 29%. *B*, Direction of motion, 161°; speed of motion, 1.5°/sec; aperture diameter, 27°; eccentricity, 10°; surround inhibition, 0%. *C*, Direction of motion, 135°; speed of motion, 12°/sec; aperture diameter, 27°; eccentricity, 15°; surround inhibition, 17%. *D*, Direction of motion, 250°; speed of motion, 8°/sec; aperture diameter, 24°; eccentricity, 11°; surround inhibition, 0%.

plotted as a function of tilt angle, with each curve corresponding to a different mean disparity of the gradient, ranging from 0.04 to 0.44°. Smooth curves are the best fits of a modified sinusoid (Eqs. 1, 2). Note that the response of the neuron is well tuned for surface tilt and that the shape of the tilt-tuning curves varies little over the range of mean disparities tested.

For a slanted plane viewed through a fixed aperture, moving the surface in depth is equivalent to shifting it within a frontoparallel plane. For this example neuron, the range of mean disparities (i.e., depths) that we tested is equivalent to shifting the center of the gradient over a range of 2° relative to the center of the RF. This allows for a considerable amount of error in centering the stimulus on the receptive field.

Figure 3 shows data from four additional MT neurons that were tested across broader ranges of mean disparities. For the neurons in Figure 3, *A* and *B*, mean disparities were chosen to straddle the peak in the disparity-tuning curve (left panels). If tilt tuning were an artifact of mis-centering the stimulus over the receptive field, then the tilt-tuning curve should undergo a phase shift of ~180 degrees for mean disparities on opposite sides of the peak. Clearly, this is not the case for either of these neurons: the shape of the tilt-tuning curve is consistent across mean disparities, although the amplitude and baseline levels of the curves vary somewhat. A similar result is seen in Figure 3*C* for a neuron that was broadly tuned to near (crossed) disparities. These neurons provide consistent signals about 3-D surface orientation across a large range of depths.

Figure 3*D* shows data that is characteristic of other neurons that we recorded (see also Fig. 8*E*). This neuron exhibits strong tilt selectivity, but the tilt-tuning curve shifts horizontally with changes in mean disparity. Although tilt preference is not invariant to changes in mean disparity, the effect is much more subtle than the 180 degrees phase shift that one would expect to see if tilt tuning were the result of poorly centering the stimulus over the receptive field of a non-tilt-selective neuron. Thus, neurons like those in Figures 3*D* and 8*E* can still provide useful signals about surface orientation. Many other MT neurons had no tilt selectivity at all (quantified below), and presumably cannot contribute to discrimination of surface orientation.

### Population analyses

To quantify the strength of tilt tuning, we equated the average response of an MT neuron to all mean disparities by vertically shifting the individual tilt-tuning curves. We then combined the data across mean disparities to create a single "grand" tilt-tuning curve. Note that this allows tilt tuning to cancel across mean disparities when the preferred tilts differ by close to 180 degrees. Thus, neurons with inconsistent tilt preferences across mean disparities will have weak tuning in the grand curve. For each neuron, we computed two metrics from this grand curve: a modulation index and a discrimination index:

$$\text{Tilt modulation index} = \frac{R_{\max} - R_{\min}}{R_{\max} - S} \tag{3}$$

$$\text{Tilt discrimination index} = \frac{R_{\max} - R_{\min}}{R_{\max} - R_{\min} + 2\sqrt{SSE/(N-M)}}. \tag{4}$$

$R_{max}$ and $R_{min}$ denote the mean firing rates of the neuron at the tilt angles that elicited maximal and minimal responses, respectively. $S$ denotes spontaneous activity. $SSE$ is the sum-squared error around the mean responses, $N$ is the total number of observations (trials), and $M$ is the number of distinct tilt values. Note that the denominator of the discrimination index incorporates a metric of response variability, whereas the modulation index does not. We present both metrics because they provide complementary information (Prince et al., 2002; DeAngelis and Uka, 2003).

Figure 4*A* shows a scatter plot of the discrimination and modulation indices for all 97 neurons in our sample, with marginal distributions along the edges of the plot. Filled symbols denote neurons for which response depended significantly on tilt ($p < 0.05$), as assessed using a two-way ANOVA with tilt angle and mean disparity as factors. By this criterion, 72% (70/97) of MT neurons are significantly tuned for surface tilt. It should be noted, however, that tilt tuning in MT is generally much weaker than either direction or disparity selectivity. The mean modulation/discrimination indices for tilt (0.29/0.42) in our sample are significantly smaller than the mean modulation/discrimination indices for both direction (0.98/0.78) and disparity (0.81/0.71) (paired $t$ test, $p \ll 0.0001$ for all comparisons). Some of this difference may be attributable to the fact that the slant was not optimized for each MT neuron and that tilt-tuning curves were combined across mean disparities, but we expect these factors to account for only a small portion of the weaker tuning to surface orientation. By varying only the disparity gradient in our stimuli, we have placed this cue to surface orientation in conflict with other cues such as texture and velocity gradients. Thus, it is also possible that tilt tuning is muted in our experiments by this cue conflict, a possibility that we cannot address at this time. In our present data set, many MT neurons exhibit clear tilt tuning, but this property is much less prominent than either direction or disparity tuning.

To quantify the consistency of tilt tuning across different mean disparities, we computed the magnitude of the difference in preferred tilt, |ΔPref. Tilt|, between all unique pairings of mean disparities for which there was significant tilt tuning (ANOVA, $p < 0.05$). For this analysis, preferred tilts were determined from the peaks of the independent sinusoid fits. Figure 4*B* shows the |ΔPref. Tilt| values for each neuron plotted as a function of the tilt discrimination index (TDI). Most neurons contribute multiple points to this plot (aligned vertically), and the largest value of

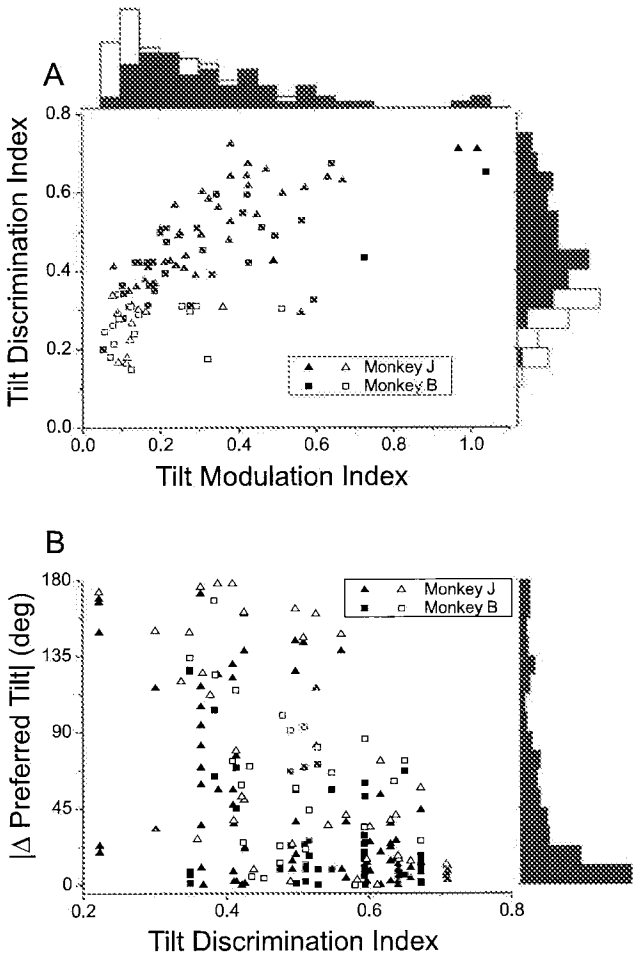


**Figure 4.** Population summary of tilt selectivity. *A*, Summary of tuning strength. The tilt discrimination index (Eq. 4) is plotted against the tilt modulation index (Eq. 3) for 97 MT neurons. Squares and triangles indicate data from monkeys B and J, respectively. Filled symbols indicate neurons for which the main effect of tilt was significant (two-way ANOVA; $p < 0.05$). Histograms along the margins of the scatter plot give distributions of the discrimination and modulation indices. *B*, Summary of the consistency of preferred tilts across mean disparities. For each neuron, we computed the absolute value of the difference in tilt preference, |ΔPref. Tilt|, between all unique pairs of mean disparities for which there was significant tilt tuning (ANOVA, $p < 0.05$). The scatter plot shows 219 |ΔPref. Tilt| values (from 64 neurons) plotted against the tilt discrimination index for each neuron. There are up to 10 data points for each neuron, aligned vertically. Open symbols show the largest value of |ΔPref. Tilt| for each neuron. The histogram (right) shows the marginal distribution of |ΔPref. Tilt|.

|ΔPref. Tilt| for each neuron is indicated by an open symbol. For neurons with large values of TDI, |ΔPref. Tilt| values are generally less than our sampling interval of 45°, indicating that tilt tuning was quite consistent across mean disparities. Correspondingly, the largest |ΔPref. Tilt| value for these well tuned neurons is also quite small. Overall, the marginal distribution in Figure 4*B* shows that 62% (135/219) of all data points correspond to |ΔPref. Tilt| values smaller than 45°. However, some neurons with low values of TDI exhibited large differences between preferred tilts at different mean disparities. The presence of |ΔPref. Tilt| values near 180° suggests that some of these neurons exhibit tilt tuning (at individual mean disparities) that is an artifact of mis-centering the visual stimulus over the receptive field. This highlights the importance of analyzing responses to multiple mean disparities straddling the peak of the disparity-tuning curve.

To determine if tilt preference was truly invariant to changes

in mean disparity, we fit the data from each neuron with two models (see Materials and Methods): one in which the tilt preference (determined by the phase and frequency of the fitted sinusoid) was allowed to vary with mean disparity, and one in which the tilt preference was constrained to be identical across mean disparities. For 25/64 neurons in Figure 4*B*, there was no significant difference between these two models (sequential $F$ test, $p > 0.05$), indicating that tilt preference was invariant to changes in mean disparity over the range tested. For many of these invariant neurons, the range of disparities tested was at least 0.8° and included disparities on both sides of the preferred disparity. We thus conclude that a substantial fraction of MT neurons code surface orientation in a depth-invariant manner.

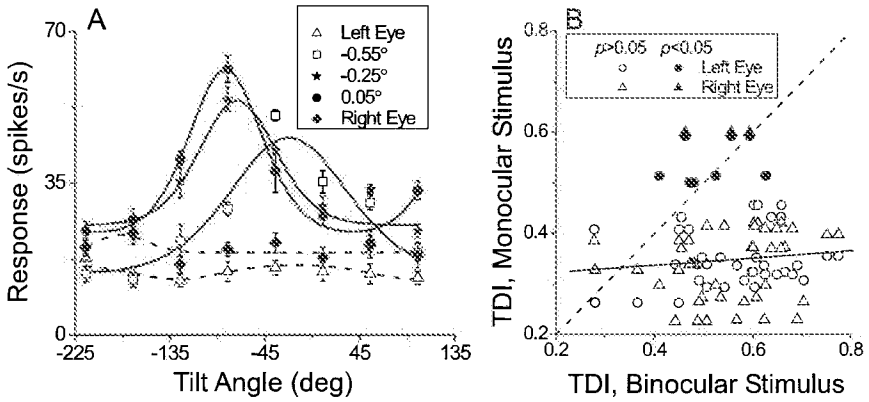


**Figure 5.** Tilt tuning does not result from monocular cues. *A*, Data from an example neuron. Solid curves show binocular tilt-tuning measurements taken at three mean disparities ranging from −0.55 to 0.05°. Dashed curves show tilt tuning for monocular controls in which only the left or right half-image was presented to the monkey. Direction of motion, 280°; speed of motion, 17°/sec; aperture diameter, 4°; eccentricity, 8.5°; surround inhibition, 72%. *B*, For each neuron tested ($n = 15$), TDI values calculated from the left-eye (circles) and right-eye (triangles) half-images are plotted against TDI values calculated from binocular stimuli at each of three mean disparities. Thus, there are 90 data points in this plot: two eyes × three mean disparities × 15 neurons. Filled symbols denote monocular controls for which tilt tuning was statistically significant (ANOVA; $p < 0.05$). The dashed line has unity slope, and the solid line is the best fit to the data using linear regression.

Tilt tuning in MT cannot be explained as an artifact of vergence eye movements. We measured the mean vergence angle of the monkey for each trial and subjected these vergence data to the same two-way ANOVA as the firing rates. Vergence angle showed a significant dependence on tilt for only 12% (12/97) of neurons. Moreover, when vergence angle was added as a covariate to the analysis of firing rates, the significance of the main effect of tilt on firing rate was unchanged for all but one of our units.

Similarly, tilt tuning does not arise from the subtle monocular dot-density cues that accompany a linear disparity gradient (see Materials and Methods). To exclude this possibility, 15 neurons were tested with left- and right-eye half-images presented separately. If tilt tuning resulted from monocular dot-density cues, then tilt selectivity should still be observed in these monocular controls. Figure 5*A* shows data from one of the neurons tested. This neuron exhibited strong tilt tuning to disparity gradients at three different mean disparities, but no significant tilt selectivity in the monocular controls. Figure 5*B* shows TDI values from monocular measurements plotted against TDI values for binocular stimuli at each of three mean disparities for each neuron (resulting in 45 data points for each eye). Only 13% of the monocular controls yielded significant tuning (ANOVA, $p > 0.05$), and there was no significant correlation between monocular and binocular measurements ($r = 0.06$; $p = 0.73$). Thus, monocular cues cannot account for tilt tuning in MT.

### Joint coding of tilt and slant

Population decoding of 3-D surface orientation signals might be more difficult if the tilt preference of single neurons varies substantially with surface slant. An alternative possibility, consistent with the joint coding of variables in other areas (e.g., orientation and spatial frequency in V1), is that tilt and slant have separable influences on the firing rate of single neurons such that slant simply modulates the strength of tuning for tilt. To examine the joint coding of tilt and slant, we obtained tilt-tuning curves at several different surface slants for a subset (29/97) of our neurons. Figure 6*A* shows a typical result. This neuron exhibited significant tilt tuning (ANOVA, $p < 0.01$) across a range of slants (from 35 to 74 degrees), with only small changes in the preferred tilt. There was no significant tilt tuning ($p = 0.14$) at a slant of 3 degrees for this neuron, and tilt tuning was weak even for the 35 degree slant.

We computed a TDI metric at each tested slant for all of the 29 neurons that were studied. Figure 6*B* summarizes how the strength of tilt tuning (TDI) varies with slant; each MT neuron is represented by four to six points in this scatter plot. There is a significant positive correlation ($r = 0.46$; $p < 0.001$) in these data, showing that tilt tuning was generally strong only for large slants. Open symbols in Figure 6*B* indicate the slant at which each neuron showed its maximal TDI. We took this as a measure of the preferred slant of each neuron because we found that peak firing rates generally varied little with slant and, thus, were an unreliable predictor of how slant modulated the tuning for tilt. Although some MT neurons prefer intermediate slants (near 45 degrees), most neurons preferred slants that were close to the largest values tested. These data appear to indicate that MT is insensitive to small slants, but there are two important caveats to be noted. First, these tilt versus slant experiments were usually done only when a neuron displayed clear tilt tuning in the initial tests with a slant of ~67 degrees. We therefore might have missed neurons that were tuned to small slants. Second, as Figure 6*B* indicates, we did not sample small slant values extensively. For these reasons, it is unclear whether there are MT neurons that are strongly tuned to small slants, and further experiments will be needed to clarify this point.

The main purpose of these tests was to determine if tilt preference was independent of slant. In Figure 6*C*, the preferred tilt of each neuron is plotted as a function of slant for all slant values that yielded significant tilt tuning (ANOVA; $p < 0.05$). Most of the curves are quite flat, indicating that there is much greater variance in preferred tilt across neurons than there is across slants for a particular neuron. In fact, neuron identity alone accounts for 90% of the variance in the data of Figure 6*B* (ANOVA), whereas adding slant as a covariate (ANCOVA) accounts for only an additional 1% of variance.

To quantify the dependence of tilt preference on slant for individual neurons, we applied the same fitting methods described above for analyzing effects of mean disparity. For the neuron in Figure 6*A* (Fig. 6*C*, open stars), independent fits to

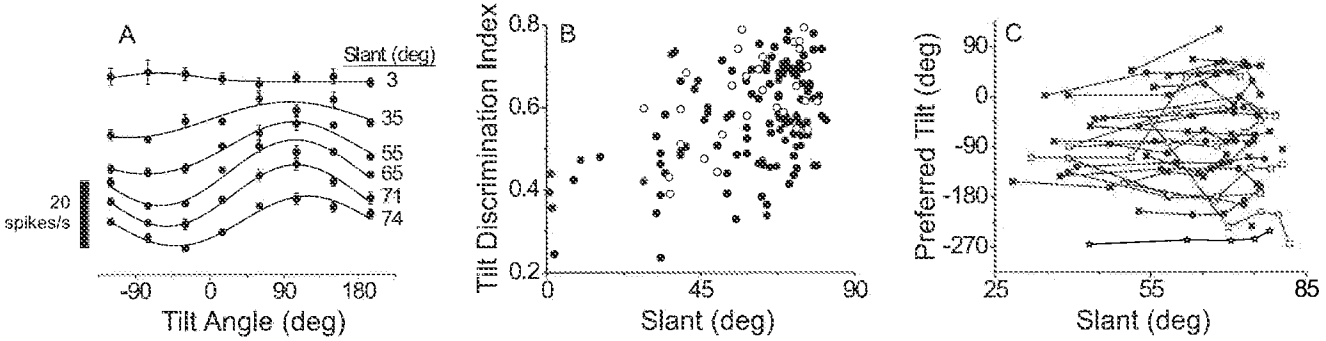


**Figure 6.** Joint coding of tilt and slant. *A*, Tilt-tuning measurements made at six different slants for the same MT neuron as in Figure 2. From top to bottom, the disparity gradient magnitudes are 0.002, 0.05, 0.1, 0.15, 0.2, and 0.25°/°. The corresponding slants are given along the right side of the plot. Smooth curves are the best fitting sinusoids (Eqs. 1, 2), and have been shifted vertically to minimize overlap and increase clarity. Calibration: 20 spikes/sec. *B*, TDI is plotted as a function of slant for all 29 neurons that were tested in the joint tilt–slant experiment. Each data point shows the TDI value for one slant, such that each neuron is represented four to six times in this plot. Open symbols indicate the slant value at which the maximum TDI was obtained for each neuron. *C*, Preferred tilt is plotted as a function of slant for the same 29 neurons. Preferred tilt values are only shown for slants at which the tilt tuning was significant (ANOVA; $p < 0.05$). Filled symbols denote neurons for which the tilt preference was statistically independent of slant (sequential $F$ test; $p > 0.05$). Stars denote data for the example neuron from *A*.

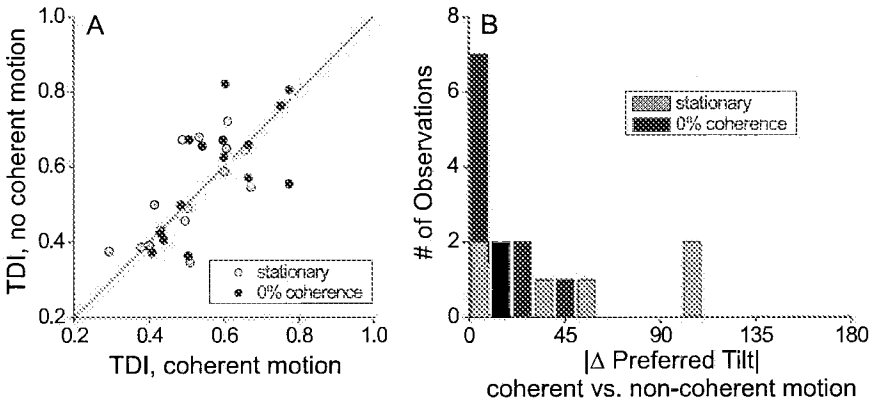


**Figure 7.** Tilt tuning does not require coherent motion. *A*, Ten MT neurons were tested (at three mean disparities each) using both coherent motion and noncoherent motion. Noncoherent stimuli were either stationary (gray symbols) or 0% coherence (black symbols). For each neuron, TDI values were computed at each mean disparity and for each motion condition; these values are compared across motion conditions in the scatter plot ($n = 30$). The solid diagonal line has unity slope. *B*, For each mean disparity that exhibited significant tilt tuning using both coherent and noncoherent motion (14/30), we computed the absolute difference between the preferred tilts, and these are plotted as a histogram. Gray and black filled bars denote the stationary and 0% coherence cases.

tilt-tuning curves at each slant were slightly, but significantly, better than fits in which the tilt-tuning curve was constrained to have the same peak and trough at each slant (sequential $F$ test; $p = 0.0007$). This example demonstrates the high sensitivity of the sequential $F$ test approach, because the variations in tilt preference across slants in Figure 6*A* are clearly quite modest. Among the 29 neurons that were tested at multiple slants, 21/29 passed the sequential $F$ test ($p > 0.05$). For this majority of neurons (Fig. 6*C*, filled symbols), the tilt preference is statistically invariant with changes in slant. We thus conclude that tilt and slant are coded in a separable manner in MT.

### Dependence on coherent motion

In the experiments described above, dots within the MT receptive field always moved with a fixed (preferred) velocity on the display screen. When a disparity gradient is applied, dots appear to stream along an oriented surface in depth. This raises the possibility that tuning for tilt and slant might simply reflect mechanisms in MT for coding 3-D velocity (i.e., motion-in-depth), although previous results from anesthetized monkeys have argued against this possibility (Maunsell and Van Essen, 1983a). To address this issue, we tested whether tilt and slant tuning are affected when coherent motion is removed from our visual stimuli. This was done either by presenting stationary dots (five neurons) or by randomly replotting the locations of dots every fourth video frame (0% motion coherence, five neurons). If tilt and slant tuning result from sensitivity to specific 3-D trajectories of the moving dots, this surface orientation dependence should be abolished when coherent motion is removed from the display. We found that this was not the case.

Figure 7*A* compares TDI values obtained using both coherent and noncoherent motion. Data are shown for 10 MT neurons, each tested at three mean disparities. Gray and black symbols denote neurons tested with stationary and 0% coherence stimuli, respectively. There is a strong correlation between TDI values for coherent and noncoherent motion (ANCOVA within-cells regression; $r = 0.69$; $p < 0.0001$), with no dependence on the type of non-coherent motion used ($p = 0.95$). Moreover, there is no significant difference between the average TDI values for coherent and noncoherent motion (paired $t$ test; $p = 0.49$). For each mean disparity with significant tilt tuning in both motion conditions, we computed the difference in preferred tilts between the coherent and non-coherent cases. Figure 7*B* shows that the tilt preferences are generally in close agreement.

These analyses show that tilt tuning does not depend on the presence of coherent motion in the receptive fields of MT neurons. Thus, tilt tuning cannot simply be a side effect of selectivity for motion-in-depth based on interocular velocity differences (Cumming, 1994). Further evidence to support coding of surface orientation rather than 3-D velocity is our finding (data not shown) that the preferred tilt axis is not correlated with the preferred (2-D) direction of motion across our population of neurons (randomization test; $p = 0.47$). Thus, it is generally not the case that MT neurons preferred the tilt angle that aligned the 2-D velocity preference with the steep slope of the disparity gradient, as might be expected if these neurons were specialized to signal the 3-D velocity of moving objects.

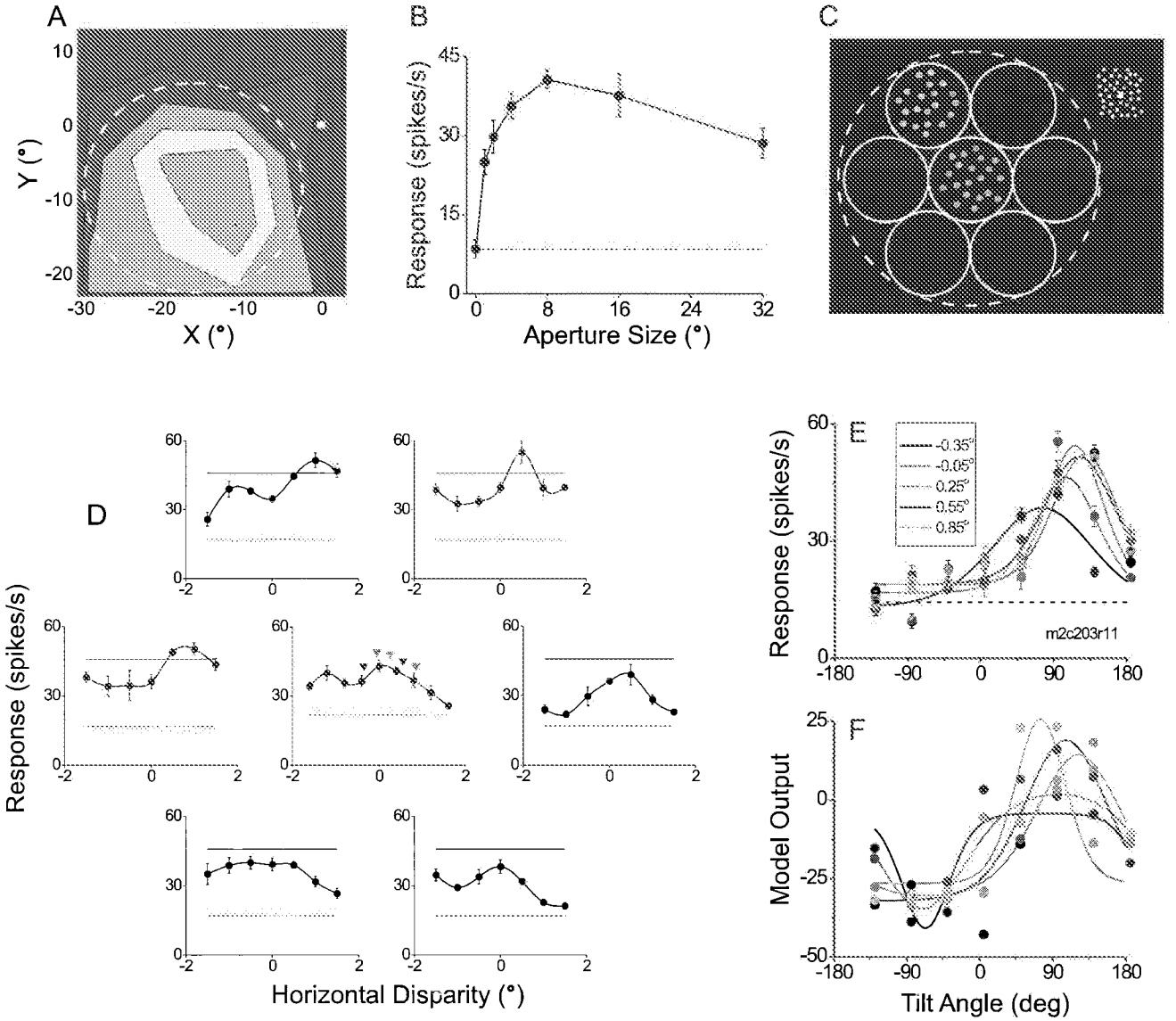


**Figure 8.** Tilt tuning can be predicted from three-dimensional receptive field substructure. All data in this figure were taken from a single MT neuron. *A*, Receptive field map, conventions as in Figure 2*C*. *B*, Size-tuning curve, conventions as in Figure 2*B*. *C*, Schematic illustration of the stimulus used to probe receptive field substructure. The receptive field (dashed circle, corresponding to that in *A*) was divided into seven subregions: a center patch containing dots at the preferred disparity, and six surrounding patches having variable disparities. A small (2°) patch of zero-disparity dots (yellow) was presented around the fixation point (white square) to help anchor vergence. *D*, Seven disparity-tuning curves are shown, corresponding to the stimulus locations in *C*. The six tuning curves around the perimeter show the disparity tuning of the neuron at each of the locations where the disparity of the surrounding patch was varied. The solid horizontal line in each of these plots shows the response of the neuron to the center patch when presented alone (at the optimal disparity). The dashed horizontal lines denote the level of spontaneous activity. The central disparity-tuning curve shows the measurement obtained with a large patch of dots that covered the entire receptive field (conventions as in Figs. 2 and 3). Arrowheads denote the mean disparities of the gradient stimulus used to test the neuron in *E*. *E*, Tilt tuning tested at five mean disparities ranging from −0.35 to 0.85° (conventions as in Fig. 3). Direction of motion, 230°; speed of motion, 2°/sec; aperture diameter, 24°; eccentricity, 19°; surround inhibition, 25%. *F*, Tilt-tuning curves predicted from a simple model based on linear summation of the responses in *D* (see in text for details).

### Receptive field mechanisms

What receptive field mechanisms might underlie the tuning of MT neurons for tilt and slant of 3-D surfaces? One possibility is that tuning for horizontal disparity varies within the classical receptive field and/or within the nonclassical surround. This is quite plausible given that MT neurons have receptive fields several times larger than their primary inputs from V1 and V2 (Albright and Desimone, 1987; Maunsell and Van Essen, 1987), allowing ample opportunity for convergence of heterogeneous disparity-tuned inputs. We therefore probed the 3-D substructure of MT receptive fields and asked whether this substructure could predict the responses to disparity gradients.

Figure 8 shows data from an MT neuron that exhibited weak conventional tuning for frontoparallel disparities (Fig. 8*D*, center panel), moderate surround inhibition (Fig. 8*B*), and strong tuning for tilt (Fig. 8*E*). The 3-D substructure of the receptive field of this neuron was probed with a stimulus array (Fig. 8*C*) consisting of a small center patch of dots presented at the preferred disparity of the neuron and six surrounding patches that had variable disparities. During each trial, the center patch was presented in conjunction with one of the surrounding patches. Because this neuron exhibited clear surround inhibition, the size of the center patch was set to the optimal size from the size-tuning curve (Fig. 8*B*), and the six surrounding patches extended into the nonclas-

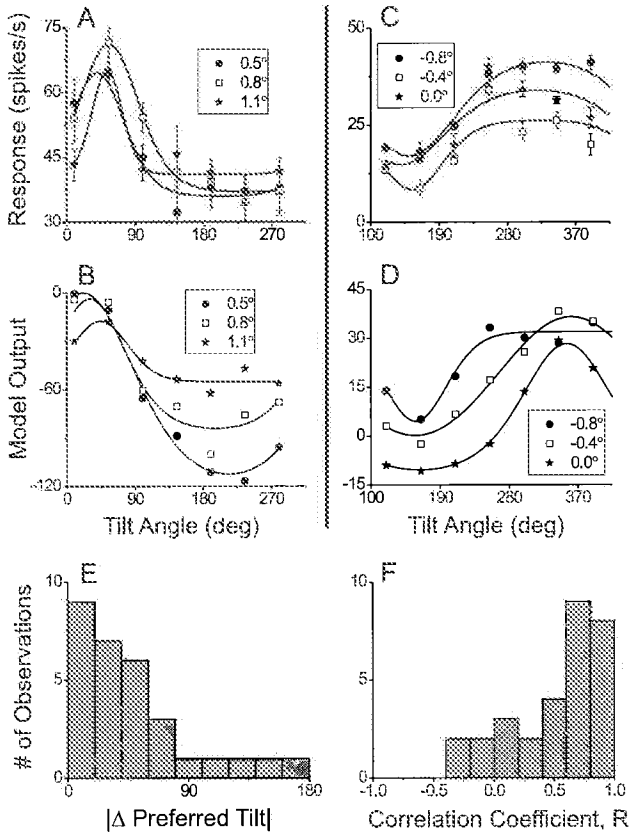


**Figure 9.** Summary of the quality of model predictions of tilt tuning. *A*, Tilt-tuning curves for an MT neuron taken at three mean disparities ranging from 0.5 to 1.1°. Direction of motion, 100°; speed of motion, 6°/sec; aperture diameter, 14°; eccentricity, 6.9°; surround inhibition, 75%. *B*, Model predictions for the neuron in *A*. *C*, Tilt tuning of a second MT neuron tested at three mean disparities ranging from −0.8 to 0.0°. Direction of motion, 255°; speed of motion, 8°/sec; aperture diameter, 26°; eccentricity, 11.2°; surround inhibition, 24%. *D*, Model predictions for neuron in *B*–*E*. Distribution of the absolute value of the difference in tilt preference, |ΔPref. Tilt|, between the predicted and the observed responses. Values of |ΔPref. Tilt| are shown for 24 mean disparities (with significant tilt tuning) from nine neurons. *F*, Distribution of correlation coefficients (*R*) between measured tilt-tuning curves and model predictions. One *R* value was computed for each of 24 means disparities from the same nine neurons as in *E*.

sical inhibitory surround. For neurons without any surround inhibition, the entire seven-patch stimulus array was presented within the classical RF (see Materials and Methods for details).

Disparity-tuning curves for each of the six surrounding locations are shown in Figure 8*D*, and it is clear that disparity tuning is not homogeneous throughout the receptive field. Maximal responses were observed at large far (uncrossed) disparities for top-left locations, whereas these disparities elicited near-minimal responses at bottom-right locations. To test whether this heterogeneity underlies tilt tuning, we crudely approximated each different disparity-gradient stimulus by an appropriate combination of disparities in these seven patches. This allowed us to predict responses of the neuron to gradients by linearly summing appropriate portions of the data in Figure 8*D*. Predicted tilt-tuning curves are shown in Figure 8*F*, and it is clear that these curves provide a good first-order prediction of the observed responses. Note, however, that the predicted responses of the model are mainly negative for this example MT neuron. This occurs because the neuron exhibits surround inhibition (Fig. 8*B*), such that responses elicited by the six surrounding patches were generally lower than responses to the center patch presented in isolation (Fig. 8*D*).

Figure 9*A* and *C* shows tilt-tuning curves (at three mean disparities) for two additional MT neurons. Figure 9*B* and *D* shows the corresponding predictions of our model based on data obtained as described in Figure 8, *C* and *D*. Because our model assumes linear summation and contains no normalization mechanisms (Britten and Heuer, 1999), one should not attempt to compare the absolute response levels of the model to those of the MT data. Rather, we emphasize that the basic shapes of the model curves, including the locations of the peaks and troughs, are quite similar for the measured and predicted tuning curves.

To quantify the quality of the model predictions, we computed the difference in preferred tilt, |ΔPref. Tilt|, between predicted and measured tilt-tuning curves. This analysis was performed on data from nine neurons that showed both strong tilt selectivity (TDI $> \approx 0.5$) and clear disparity selectivity in the seven-patch mapping experiment (average DDI across the six locations $\geq 0.5$). For neurons with weak tilt tuning or weak disparity modulation, we found that model predictions were very noisy. Figure 9*E* shows the histogram of |ΔPref. Tilt| for 24 mean disparities from these nine data sets. Only mean disparities with significant tilt tuning (ANOVA; $p < 0.01$) were included in this analysis. Most of the differences in preferred tilts (60%) were smaller than 45 degrees, and very few were larger than 90 degrees, indicating that tilt preferences were generally well matched between measured and predicted tuning curves. We also calculated the correlation coefficient ($R$) between measured and predicted tuning curves for each mean disparity. Figure 9*F* shows the distribution of these correlation coefficients. Most values are >0.5, indicating that predicted and measured tuning curves typically had quite similar shapes. Together, these results indicate that the tilt selectivity of MT neurons can be primarily explained by variations in local disparity tuning within the MT receptive field.

### Involvement of surround inhibition

Previous computational and physiological studies have reported that spatially asymmetric surround inhibition is essential for generating the selectivity of MT neurons to surface orientation defined by speed gradients (Buracas and Albright, 1996; Xiao et al., 1997). Is surround inhibition also necessary for generating the tilt selectivity that we have observed? Among the nine neurons analyzed in Figure 9, *E* and *F*, five showed some surround inhibition, whereas four neurons showed no surround inhibition at all. For the latter neurons, heterogeneous disparity tuning within the classical RF was sufficient to predict tilt preference. This observation suggests that surround inhibition is not a primary determinant of tilt selectivity in our experiments, but this conclusion is tenuous based on only nine neurons. To clarify the role of surround inhibition, we examined how tilt selectivity depends on both the strength and spatial distribution of surround inhibition for our full sample of neurons.

The overall strength of surround inhibition was determined from size tuning curves (Figs. 2*B*, 8*B*) by computing the percentage of surround inhibition:

$$\%\ \text{Surround inhibition} = 100 \times \left( \frac{R_{opt} - R_{largest}}{R_{opt} - S} \right), \quad (5)$$

where $R_{opt}$ is the response to the optimal stimulus size, $R_{largest}$ is the response to the largest stimulus, and $S$ denotes the level of spontaneous activity. These values, as well as the statistical significance of surround inhibition, were determined from curve fits to size tuning curves as described elsewhere (DeAngelis and Uka, 2003). Figure 10*A* shows the TDI plotted against percent of sur-

round inhibition for our population of 97 MT neurons. Filled symbols indicate neurons with significant surround inhibition ( $p < 0.05$). We find no significant correlation between the strength of surround inhibition and the strength of tilt selectivity ($r = 0.006$; $p = 0.95$), indicating that surround inhibition is not necessary for tilt tuning in MT.

To assess whether tilt selectivity depends on the spatial distribution (i.e., asymmetry) of surround inhibition (Xiao et al., 1997), we analyzed responses from 37 neurons that were tested using the seven-patch stimulus configuration of Figure 8*C*. For each of the six surrounding patch locations, we computed the average response of the MT neuron across disparities, and we plotted a vector having the average response as its length and the location of the patch as its direction. We then computed the vector average across all six patch locations to get an estimate of surround asymmetry. Specifically, we construct a surround asymmetry index as the magnitude of the vector average divided by the average magnitude of the individual vectors. This index will be close to zero if responses to the surrounding patches are symmetric about the receptive field center. Larger values of the index indicate stronger spatial asymmetry in responses to the surrounding patches. Figure 10*B* shows TDI values as a function of the surround asymmetry index for 37 MT neurons. We find no significant correlation between these variables ($r = 0.02$; $p = 0.89$), indicating that tilt tuning does not depend on asymmetric surround effects.

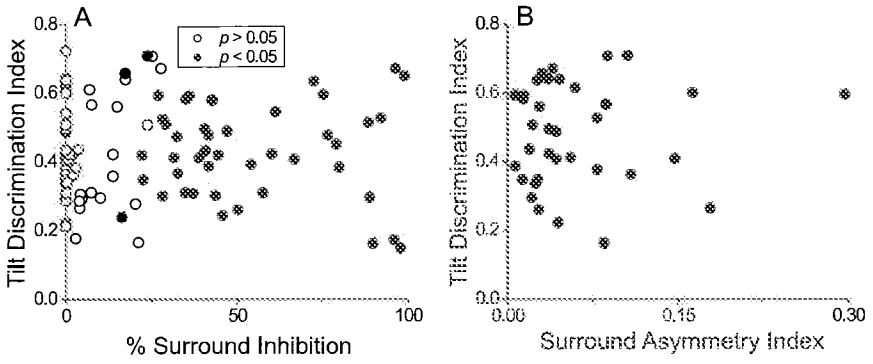


**Figure 10.** Tilt tuning strength is not correlated with either the strength or spatial asymmetry of surround inhibition. *A*, The TDI is plotted against the percentage of surround inhibition for the 97 MT neurons in our sample. Neurons with significant surround inhibition are indicated by filled symbols ( $p < 0.05$). *B*, TDI is plotted against the surround asymmetry index (see text) for 37 MT neurons that were tested with the stimulus configuration of Figure 8*C*.

For 44/97 neurons, we measured tilt-tuning curves using two different stimulus sizes, randomly interleaved. The large size was chosen as described in Materials and Methods, whereas the small size was twofold to threefold smaller. Thus, for neurons with surround inhibition, the small size was near-optimal as given by the size-tuning curve. For neurons without surround inhibition, the small size was one-third to one-half the size of the classical RF. For both groups of neurons, TDI values were significantly greater (*t* test; $p < 0.01$) for the large stimulus than for the small stimulus (the percentage difference was 24% for neurons with surround inhibition, 19% for neurons without surround inhibition).

Together, the data of Figures 8–10 indicate that tilt tuning in response to disparity gradients depends mainly on heterogeneity of disparity tuning within the receptive fields of MT neurons (including the nonclassical surround), not on the presence or spatial distribution of surround inhibition. Further work will be necessary to fully understand the 3-D organization of MT receptive fields.

## Discussion

Most models of cortical visual processing have focused on the roles that area MT plays in computing motion within frontoparallel planes (Nowlan and Sejnowski, 1995; Wang, 1997; Simoncelli and Heeger, 1998; Koechlin et al., 1999; Perrone and Thiele, 2002) (but see Lappe, 1996; Buracas and Albright, 1996). Recently, it has been demonstrated physiologically that area MT contributes to depth judgments involving frontoparallel surfaces (DeAngelis et al., 1998) and that integration of motion and disparity signals allows MT neurons to signal the perceived depth-ordering of transparent surfaces (Bradley et al., 1995, 1998; Dodd et al., 2001; Grunewald et al., 2002). We now show that MT contains robust, disparity-based signals regarding the 3-D orientation (tilt and slant) of planar surfaces. This tilt selectivity does not result from vergence eye movements or subtle monocular dot-density cues, and approximately one-half of MT neurons respond more strongly to a tilted stimulus (i.e., a nonzero slant) than to any frontoparallel stimulus of the same size (data not shown). In addition, we show that the tilt preference of MT neurons is primarily independent of the mean depth and slant of the surface, properties that may simplify the extraction of 3-D orientation signals from a population of MT neurons. Together, these findings show that the visual representation in MT is more complex than previously thought; it contains information not only about the local velocity of features on the retina, but also about the 3-D structure of the environment from which those velocity signals arise.

Although we have shown that MT neurons carry information about the 3-D orientation of planar surfaces, this selectivity could arise because of other computations in MT. Tilt and slant tuning could be a by-product of selectivity for 3-D velocity (motion-in-depth), which can be computed via either interocular velocity differences or changes in binocular disparity over time (Cumming, 1994). Our control experiments and analyses suggest that this is unlikely, for two main reasons. First, tilt and slant tuning remain unchanged when coherent motion is removed from our stimuli, thus excluding the possibility that tilt tuning reflects the calculation of motion-in-depth based on interocular velocity differences. Second, we found no consistent relationship between the preferred tilt of MT neurons and their preferred 2-D velocity. For an object moving in 3-D space, binocular disparity changes over time along the 3-D vector of the movement. As a result, the direction of maximal slope of the gradient is aligned with the 2-D velocity of the object. If MT neurons were specialized to code 3-D velocity, then we might expect their gradient preference to be similarly aligned with their preferred 2-D velocity (e.g., a neuron preferring rightward 2-D motion would have a preferred tilt of 0 or 180 degrees, whereas a neuron preferring upward motion would prefer a tilt of 90 or 270 degrees) (Fig. 1). Some MT neurons behave this way, but most do not. Thus, our findings suggest that gradient selectivity in MT plays a more general role in the analysis of 3-D scene structure. This conclusion is consistent with that of a previous study in the anesthetized monkey, where a specialization for coding of motion-in-depth was not found in MT (Maunsell and Van Essen, 1983a).

We have shown that the tilt preference of MT neurons can be predicted from heterogeneity of disparity tuning within the clas-

sical receptive field and/or the nonclassical surround. Although the quality of our model predictions is far from ideal, we think their accuracy is striking given the simplicity of the model and the coarseness of our measurements of receptive field substructure. These results suggest that tilt selectivity arises from a combination of inputs with disparity preferences that vary systematically across space within the MT receptive field. The details of the mechanisms that underlie this pooling remain unclear, and our data do not allow us to evaluate whether nonlinear interactions are involved. Our model was based on linear summation of responses to the different stimulus patches (Fig. 8*C*), but each surrounding patch was always presented in conjunction with the center patch. Thus, our data (Fig. 8*D*) may include nonlinear interactions between the center and surrounding patches. Further experiments will be needed to clarify the mechanisms underlying tilt selectivity.

Our findings complement and extend those of a few previous studies of disparity-based surface representation. Taira et al. (2000) reported that neurons in the CIP are selective for the tilt of planar surfaces specified by disparity gradients, although they did not sufficiently exclude the possibility that these responses arose through monocular cues, variations in vergence angle, or from inaccurate centering of stimuli over the receptive fields of the neurons. It is also difficult to determine if tilt selectivity is more or less common in CIP than in MT because there is no quantitative summary of tilt selectivity in the Taira et al. (2000) study. In any case, our findings show that disparity-gradient signals arise substantially earlier in the visual hierarchy than the parietal lobe. MT receives direct input from V1 and V2 (Maunsell and van Essen, 1983b), whereas CIP is thought to be two or three synapses removed from these areas (Sakata et al., 1997). Our findings confirm expectations from psychophysical and theoretical considerations that 3-D surface orientation should be coded early in the visual pathways (Gibson, 1950; Marr, 1982; Nakayama, 1996).

Recently, Hinkle and Conner (2002) have reported the presence of 3-D orientation tuning in macaque area V4, indicating that 3-D orientation signals are present midway along both the dorsal and ventral processing streams. A few differences between their study and ours (other than the brain area) are worth noting. First, because Hinkle and Conner (2002) used bar stimuli, tilt was confounded with 2-D orientation in their stimuli. Thus, they clearly demonstrate the presence of slant selectivity in V4, but one cannot draw any conclusions about tilt tuning or about the joint coding of tilt and slant. Second, Hinkle and Conner (2002) did not find slant tuning for textured surface stimuli (like ours) that lacked orientation cues. Thus, V4 neurons do not appear to be coding surface orientation from the gradient of horizontal disparities, but may instead be dependent on orientation disparities. V4 and MT may therefore contain different mechanisms for signaling 3-D orientation.

Our findings dovetail nicely with previous studies showing that the response of MT neurons depends on the spatial orientation of speed gradients (Treue and Andersen, 1996; Xiao et al., 1997), which may also serve as a cue to the tilt and slant of 3-D surfaces. It should be noted, however, that the mean speed of the stimuli was not varied in these studies to control for the possibility that gradient selectivity depends on stimulus centering. Also, the tuning of MT neurons to speed gradients appears to depend on the presence of asymmetric surround inhibition (Buracas and Albright, 1996; Xiao et al., 1997), whereas we did not find any consistent relationship between the strength of tilt tuning and the strength or asymmetry of surround inhibition (Fig. 10). Despite these differences, the combination of these studies suggests that MT neurons may integrate information from disparity gradients, velocity gradients, and perhaps other cues to provide robust estimates of 3-D surface orientation. We are currently testing this hypothesis.

This work adds to a small, but rapidly growing, body of work on the neural coding of higher-level disparity signals that underlie perception of 3-D structure (Shikata et al., 1996; Bradley et al., 1998; Eifuku and Wurtz, 1999; Janssen et al., 1999, 2000; Taira et al., 2000; von der Heydt et al., 2000; Dodd et al., 2001; Hinkle and Connor, 2002; Thomas et al., 2002). Our results reveal a new aspect of the depth representation found within area MT and provide new support for the idea that MT plays a role in the analysis of 3-D scene structure. Additional studies can now be focused on mapping the 3-D substructure of MT receptive fields, probing for causal links between MT activity and surface perception, and exploring how MT neurons integrate multiple cues to surface orientation. Along with similar studies in other areas, this endeavor should reveal the neural mechanisms that underlie our impressive ability to see the world in three dimensions.

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# Exhibit D2

# Effect of Obesity on Parameters of Ovarian Reserve in Premenopausal Women

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## Abstract

**Objective:** To study the relationship between obesity and serum and ultrasound markers of ovarian reserve in premenopausal women.

**Methods:** We performed a cross-sectional comparative study of two age-matched groups of premenopausal participants (early transition phase): 50 participants ("non-obese") had a BMI < 30 kg/m$^2$, and the other 50 participants ("obese") had a BMI of 30 to 35 kg/m$^2$. The obese women had a mean age of 46.2 years and the non-obese women had a mean age of 46.1 years. Blood samples were collected from all participants, anthropometric measurements were calculated, and transvaginal ultrasonography was performed to measure the antral follicle count (AFC) and ovarian volume during the early follicular phase. The blood samples were assayed for antimüllerian hormone (AMH), follicle-stimulating hormone (FSH), fasting blood glucose (FBG) and two-hour postprandial blood glucose (2h PP).

**Results:** There was no significant difference between the two groups in mean age, levels of serum AMH, serum FSH, FBG, 2 hr PP, or AFC. Ovarian volume was significantly lower in obese women (3.7 ± 0.8 mL) than in non-obese women (6.6 ± 0.4 mL) ($P$ = 0.03). There was no significant correlation between BMI and serum AMH, serum FSH, FBS, or 2 hr PP.

**Conclusion:** Obesity has no association with levels of serum FSH, AMH, blood glucose, or AFC indicating that obesity is unlikely to affect ovarian reserve in the perimenopausal age group.

## Résumé

**Objectif :** Étudier la relation entre l'obésité et les marqueurs sériques et échographiques de la réserve ovarienne chez les femmes préménopausées.

**Méthodes :** Nous avons mené une étude comparative transversale portant sur deux groupes de participantes préménopausées (phase de transition précoce) appariées en fonction de l'âge : 50 participantes (« non obèses ») présentant un IMC <30 kg/m$^2$ et 50 autres participantes (« obèses ») présentant un IMC se situant entre 30 et 35 kg/m$^2$. L'âge moyen des femmes obèses était de 46,2 ans et celui des femmes non obèses était de 46,1 ans. Des échantillons sanguins ont été prélevés chez toutes les participantes, leurs mesures anthropométriques ont été calculées et une échographie transvaginale a été menée pour mesurer la numération des follicules antraux (NFA) et le volume ovarien au cours de la phase folliculaire précoce. Les échantillons sanguins ont fait l'objet d'un titrage visant l'hormone antimüllérienne (HAM), l'hormone folliculo-stimulante (FSH), la glycémie à jeun (GJ) et la glycémie postprandiale à deux heures (2 h PP).

**Résultats :** Nous n'avons constaté aucune différence significative entre les deux groupes en matière d'âge moyen, de taux sérique d'HAM, de taux sérique de FSH, de GJ, de 2 h PP ou de NFA. Le volume ovarien était considérablement plus faible chez les femmes obèses (3,7 ± 0,8 ml) que chez les femmes non obèses (6,6 ± 0,4 ml) (P = 0,03). Nous n'avons constaté aucune corrélation significative entre l'IMC et le taux sérique d'HAM, le taux sérique de FSH, la GJ ou la 2 h PP.

**Conclusion :** L'obésité n'est aucunement associée au taux sérique de FSH, au taux sérique d'HAM, à la glycémie ou à la NFA, ce qui indique qu'il est peu probable que l'obésité affecte la réserve ovarienne au sein du groupe d'âge périménopausique.

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**Key Words:** Antimüllerian hormone, AMH, follicle stimulating hormone, FSH, body mass index, BMI, obesity, antral follicle count, ovarian volume, ovarian reserve

Competing Interests: None declared.

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## INTRODUCTION

According to a global survey, more than 30% of women in the group aged 25 to 44 years are overweight (BMI 25 to 30 kg/m$^2$), and 20% are obese.[1] In addition to conditions such as diabetes mellitus, hypertension, cardiovascular disease, pancreatitis, and musculoskeletal diseases, obese women are more likely to experience reproductive problems.[2] Overweight women, as distinct from obese women, are known to be at higher risk of menstrual dysfunction and anovulation, possibly due to altered secretion of pulsatile GnRH.[3] Obese women, even those with regular menstrual cycles, have been found to have reduced fecundity.[4]

In women undergoing assisted reproductive technology, being obese or overweight has been associated with a need for higher doses of gonadotropins, increased cycle cancellation rates, and fewer oocytes retrieved than in women of normal weight.[5] Lower rates of embryo transfer, pregnancy, and live birth have also been reported in these women, as have higher miscarriage rates.[5,6] However, other studies

have not found any negative effect of obesity on ART outcome.[7,8]

The term "ovarian reserve" refers to the quantity and quality of a woman's current reservoir of oocytes, and is closely associated with reproductive potential. It is an indirect measure of a woman's reproductive age.[9] Over the past two decades, a number of tests of ovarian reserve have been used to determine follicle number and quality and to predict the outcome of assisted reproduction procedures.[10] The woman's age and assays of serum FSH in the early follicular phase were among the earliest and most useful parameters used for evaluation of ovarian reserve.[11,12] Several ultrasound parameters have been used for evaluation of ovarian reserve, including ovarian volume[13,14] and the antral follicle count, with varying degrees of reliability.[15,16]

Recently, serum antimüllerian hormone levels have been introduced as a novel measure of ovarian reserve. AMH is a product of the granulosa cells in preantral and antral follicles.[17] Serum AMH levels decline with age and are correlated with the number of antral follicles and the ovarian response to hyperstimulation.[18,19]

Few studies have evaluated the effect of obesity on ovarian reserve. Some studies have identified lower AMH levels in obese women than in non-obese women.[20,21] However, no effect of obesity on the AFC has been identified.[21]

We conducted the present study to examine the effect of obesity on ovarian reserve in women in the late reproductive age group. We assessed the effect of obesity on accepted markers of ovarian reserve, specifically levels of basal FSH and AMH, as well as the ultrasound parameters ovarian volume and AFC.

## METHODS

This study was conducted in the Department of Obstetrics and Gynecology, Kasr Al-Ini hospital, Cairo University between June 2008 and July 2009. All participating women gave written informed consent before beginning the study.

We included 50 participants with a BMI of 30 to 35 kg/m$^2$ (group A, obese women) and 50 age-matched participants with BMI < 30 kg/m$^2$ (group B, non-obese women) serving as control group. All the women were in the early transition phase of the late premenopausal state. According to the staging system for reproductive aging in women, this phase is characterized by regular menstrual cycles of between 22 and 35 days, with variability in cycle length ≥ 7 days in either direction compared with patient's baseline and observed for at least two cycles.[22]

To meet the inclusion criteria, women had to be in the late premenopausal stage with an intact uterus and ovaries and to have had regular menstrual cycles for the previous three months. Exclusion criteria were current use of hormones or drugs that may affect ovarian function, smoking, pregnancy, lactation, hysterectomy, previous ovarian surgery, clinical or ultrasound criteria suggesting polycystic ovarian syndrome or endometriosis, or any medical condition that might affect ovarian function.

All participating women underwent a comprehensive history and thorough physical examination, calculation of BMI, assays of serum FSH and AMH, measurement of fasting blood glucose and 2 hr PP, and had a transvaginal ultrasound examination for assessment of AFC and ovarian volume.

For calculation of BMI, height and weight were measured using the same scale for all participants. Blood samples were withdrawn from the antecubital vein on cycle day 2, 3, 4, or 5 of the menstrual cycle in all women. All samples were centrifuged at 2000 g for 15 minutes. Serum was separated and stored at –20 °C until assayed.

Measurement of serum FSH was performed using a micro-particle enzyme immune assay (Architect 2000 FSH, Abott Diagnostic, Germany). Serum levels of AMH were determined by enzyme-linked immunosorbent assays using commercial kits from Diagnostic Systems Laboratories (DSL Inc., Webster, TX) according to the manufacturer's instructions. The detection limits of this assay were 0.017 ng/mL, and its intra-assay and inter-assay coefficients of variation were 4.6% and 8.0% respectively.

Transvaginal ultrasound was performed during the early follicular phase (cycle day 2, 3, or 4), by means of a transvaginal ultrasound scanner (Accuvix XQ, Medison Co. Ltd., Seoul, Korea) with a 4 to 7 MHz probe. In each ovary, the total number of small follicles (2–10 mm) was counted. The total follicle count was the sum of the follicle counts in each ovary.

Ovarian volume was measured by three-dimensional ultrasonography. After visualizing the ovaries in a sagittal two-dimensional plane, the 3-D mode was activated; the ovary was scanned in slow sweep mode to obtain good resolution .The technique was used to obtain a sequence of six longitudinal sections of each ovary around a fixed axis, each after a 30° rotation from the previous section. The acquired 3-D volume was stored immediately using the Sonoview program (Sonoview, Medison Co. Ltd. Seoul, Korea) until it was included for analysis. The acquired volume dataset was analyzed using the Virtual Organ Computer-aided Analysis

ABBREVIATIONS

| | |
|---|---|
| AFC | antral follicle count |
| AMH | antimüllerian hormone |
| ART | assisted reproductive technology |
| FBG | fasting blood glucose |
| FSH | follicle stimulating hormone |
| LH | luteinizing hormone |

imaging program (VOCAL, Medison Co. Ltd., Seoul, Korea), and the volume of each ovary in mL was calculated. Mean ovarian volume was the average of the calculated volume of the left and right ovary.

Different outcome measures were compared using Student *t* test, Mann-Whitney test, or Fisher exact test as appropriate. Significant relationships among study parameters were evaluated by Pearson correlation coefficient. *P* values $< 0.05$ were considered to be significant. Statistical analysis was performed using Arcus Quickstat version I (Research Solutions, Cambridge, UK).

Ethical approval for the study was provided by Kasr Al-Ini Hospital Research Advisory Board.

## RESULTS

The 50 women in group A (obese women) had a mean BMI of 32.9 kg/m$^2$, with a range of 30 to 35 kg/m$^2$, and the non-obese women had a mean BMI of 25.6 kg/m$^2$, with a range of 24 to 29 kg/m$^2$. The mean age in the obese group was 46.2 years; median age was 46.25 years, with a range of 40 to 48 years. The mean age in the non-obese group was 46.1 years; median age was 46 years, with a range of 38 to 48 years. The mean BMI in the obese group (32.9 ± 1.32) was significantly higher than that of the non-obese group (25.6 ± 1.36) ($P < 0.01$). There was no significant difference between the two groups regarding age, serum levels of AMH or FSH, FBG, 2 hours post prandial, or AFC. Mean ovarian volume was significantly lower in obese women (3.7 ± 0.8 mL) than in non-obese women (6.6 ± 0.4 mL) ($P = 0.03$). These data are shown in the Table.

There was no significant correlation between BMI and serum AMH, serum FSH, FBG, and 2 hours post prandial.

**The studied parameters in the two groups**

| | Group A (obese women) | Group B (non-obese women) | *P* |
|---|---|---|---|
| Age (years) | 46.2 ± 6.4 | 46.1± 3.3 | 0.18 |
| BMI (kg/m$^2$) | 32.9 ± 1.32 | 25.6 ± 1.36 | 0.01 |
| AMH (ng/ml) | 2.55 ± 0.81 | 3.39 ± 0.24 | 0.56 |
| AFC | 4.36 ± 0.11 | 5 ± 0.31 | 0.32 |
| FSH (IU/L) | 12.2 ± 4.8 | 13.1 ± 2.1 | 0.71 |
| FBS (mg/dL) | 98.5 ± 18.3 | 103.4 ± 11.9 | 0.59 |
| 2 hours post prandial (mg/dL) | 121.2 ± 29.2 | 122.6 ± 23.5 | 0.91 |
| Ovarian volume (mL) | 3.7 ± 0.8 | 6.6 ± 0.4 | 0.03 |

Values are expressed as mean and SD

## DISCUSSION

Changes in body fat and its distribution occur during the menopausal transition,[23] but the health-related implications for these body composition changes are ill-defined. It remains to be determined if the change in body fat will affect the endocrine milieu of women in the late reproductive age.

Our study was designed to explore the effect of obesity on ovarian reserve in late reproductive age women. We studied the effect of obesity on serum and ultrasound markers of ovarian reserve, and because of a possible effect on folliculogenesis of abnormal blood glucose homeostasis associated with obesity,[24,25] we evaluated blood glucose levels in participating women to detect a possible effect on ovarian function.

Several studies have suggested a negative effect of obesity on parameters of ovarian reserve. De Pergola et al.[25] suggested that overweight and obese fertile women, in comparison with women of normal weight, have lower serum levels of FSH, LH, inhibin B, and estradiol in the early follicular phase, with a possible direct inhibitory effect of body mass on gonadotropin and estradiol production, independent of age and insulin activity. Other investigators reported lower levels of AMH in obese women compared with normal weight women in the late reproductive age.[20,21] However, these studies documented that obesity had no effect on ovarian follicle count. They suggested that lower levels of AMH in obese late reproductive age women result from physiologic processes other than decreased ovarian reserve.[20,21,25]

Our results showed that there are no significant differences in serum levels of AMH, FSH, blood glucose, and AFC between obese and non-obese women. There was no significant correlation between BMI and the serum or ultrasound markers of ovarian reserve. Accordingly, we are suggesting that obesity may have limited effect on ovarian reserve in late reproductive age women.

The fact that our results showed no effect of obesity on AMH levels, contrary to other studies, may be related to factors in our study population and limitations in other reports. Our group of obese women was limited to women with a BMI between 30 and 35 kg/m$^2$. We did not include morbidly obese patients because we thought that this specific group of women may have a different endocrine profile that may not apply to women with lesser obesity. Moreover, in our population morbidly obese patients often have comorbid conditions in the late reproductive age group that may affect ovarian function. We included overweight women (with a BMI between 25 and 30 kg/m$^2$) in the control group of non-obese women.

It is important to note that the serum FSH levels in both the study group and the control group were in the high range, reflecting borderline ovarian reserve. The effect of obesity may not have been apparent in these women because of the limited ovarian reserve.

Our findings may have been different if the study women had been younger. Freeman et al.[20] recently provided

evidence suggesting a negative effect of obesity on AMH levels, but the fact that 21% of their cohort was postmenopausal with undetectable levels of AMH may have skewed their findings. In a study of women with polycystic ovary syndrome by Pigny et al.,[26] the AMH levels were lower in obese than non-obese women, but the difference was not statistically significant. Another study suggested no correlation between BMI and AMH in women with polycystic ovary syndrome and control subjects.[27] These data may support our findings. We did not find an effect of obesity on AFC, which has been suggested by others.[21,25] This supports our impression of a limited effect of obesity on ovarian reserve.

Another controversial point is the relationship between BMI and ovarian volume. We found that ovarian volume was significantly less in obese than in non-obese women. This finding was supported by the study by Zaidi et al.,[28] which showed a significant negative correlation between ovarian volume and BMI in older fertile women only. However, ovarian volumes did not differ by body size according to another study.[21] The different findings could be related to different population characteristics, and different techniques used to measure and calculate the ovarian volume. Moreover, ovarian volume measurement may be less accurate among obese women because of restricted ultrasound imaging.

Our findings do not support an effect of obesity on the selected parameters of ovarian reserve among our cohort of premenopausal women. However, this should be verified by larger studies with clear distinctions between normal, overweight, obese, and morbidly obese women, and between groups of different age and menopausal status.

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# Exhibit E

Z. Immun.-Forsch. Bd. 148, S. 285–290 (1975)

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# Serum IgD and IgE in Rheumatoid Arthritis

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## Abstract

Serum immunoglobulins IgD and IgE have been determined by a single radial immunodiffusion technique and a radioimmunoassay method in serum samples from 95 rheumatoid patients, 5 subjects with Sjögren's syndrome and 50 healthy controls, and compared with levels of IgG, IgM and IgA fractions measured in the same subjects. The IgD and IgE serum content resulted similar in the rheumatoid, Sjögren's and control sera. No correlation of IgD and IgE values with changes of other immunoglobulins or with the activity and the duration of the rheumatoid disease was observed.

## Introduction

Several authors (6, 23, 33) measured immunoglobulins concentration in patients with rheumatoid arthritis (RA) and found they had significantly higher IgG and IgA serum levels than controls. IgD and IgE constitute classes of human immunoglobulins which are distinct from IgG, IgA and IgM fractions (15, 17, 29). The measurement of IgD and IgE concentrations might contribute to a better knowledge of the role and the involvement of these immunoglobulins in hypersensitivity phenomena and in other immune responses. The purpose of the present investigation was to study the IgD and IgE behaviour in RA in order to ascertain whether noticeable increases of these fractions are observable in this disease, as expression of a polyclonal gammopathy, and the eventual relationship with the activity and the duration of the disease.

## Materials and Methods

IgD and IgE serum concentrations were determined in 50 volunteer control subjects without signs of illness, in 5 subjects with Sjögren's syndrome and in 95 patients with definite or classical RA, clinically and radiologically ascertained according to the ARA criteria, with a positivity for latex-test varying from 1 : 40 to 1 : 2560 and for Waaler-Rose from 1 : 32 -1 : 2048. The control group was age matched with study group. For comparison, also serum levels of IgG, IgA and IgM were examined in all subjects. Quantitative estimation of IgD, IgG, IgA and IgM was made by radial diffusion in agar plates (immunodiffusion plates:

Meloy Laboratories, Springfield/Virginia) using the method of Mancini and others (22). The diameters of the precipitin rings were measured after 24 hours of incubation at room temperature and were recorded as blindtest. A series of dilutions standard was used to calibrate every plate. Serum IgE levels were measured by a radioimmunosorbent test (Phadebas IgE test, Pharmacia Laboratories) utilizing anti-IgE antibodies covalently bound to Sephadex particles as a solid phase reactant. This test is relatively simple and is accurate to IgE concentrations as low as 10 ng/ml. IgG, IgA and IgM levels were expressed as mg/ml. IgD values as mg per 100 ml, while IgE concentrations were expressed as ng/ml.

The presence of rheumatoid factor (RF) was investigated in all sera by the latex-agglutination slide test (Hyland Laboratories, Los Angeles/California) and by Waaler-Rose technique modified according to Valkenburg (32).

## Results

The quantitative results are shown in Table 1 and 2. In patients with RA the mean level of IgG, IgA and IgM resulted respectively of $16.33 \pm 5.70$, $3.70 \pm 2.20$, and $1.24 \pm 0.70$ mg/ml in comparison to values of $16.50 \pm 6.20$, $3.58 \pm 1.80$, and $1.18 \pm 0.90$ obtained in Sjögren's patients and of $12.15 \pm 2.36$, $2.87 \pm 1.10$, and $1.20 \pm 0.85$ mg/ml observed

Tab. 1. IgG-, IgA-, IgM-immunoglobulin concentrations

| Ig type | Serum sample | Mean immunoglobulin concentration ± S.D. (mg/ml) |
|---|---|---|
| IgG | Normal subjects | 12.15 ± 2.36 |
| | Sjögren's syndrome | 16.50 ± 6.20 |
| | Rheumatoid patients | 16.33 ± 5.70 |
| IgA | Normal subjects | 2.87 ± 1.10 |
| | Sjögren's syndrome | 3.58 ± 1.80 |
| | Rheumatoid patients | 3.70 ± 2.20 |
| IgM | Normal subjects | 1.20 ± 0.85 |
| | Sjögren's syndrome | 1.18 ± 0.90 |
| | Rheumatoid patients | 1.24 ± 0.70 |

Tab. 2. IgD- and IgE-immunoglobulin concentrations

| Ig type | Serum sample | Mean immunoglobulin concentration ± S.D. |
|---|---|---|
| IgD (mg/100 ml) | Normal subjects | 5.8 ± 5.5 |
| | Sjögren's syndrome | 4.8 ± 3.6 |
| | Rheumatoid patients | 5.5 ± 4.2 |
| IgE (ng/ml) | Normal subjects | 250 ± 110 |
| | Sjögren's syndrome | 210 ± 95 |
| | Rheumatoid patients | 280 ± 150 |

in the group of control subjects. This finding confirms the previously reported data (23). IgD and IgE levels of rheumatoid and Sjögren's sera did not differ significantly from those of control subjects. The mean serum IgD of rheumatoid patients was $5.5 \pm 4.2$ mg/100 ml, compared to the Sjögren's patients mean of $4.8 \pm 3.6$ and to the healthy controls mean of $5.8 \pm 5.5$ mg/100 ml. The mean concentration of IgE resulted of $250 \pm 110$ ng/ml in the group of controls, $210 \pm 95$ ng/ml in Sjögren's patients and $280 \pm 150$ ng/ml in subjects with RA. These differences are not statistically significant.

## Discussion

The results obtained suggest that normal concentrations of IgD and IgE may occur in RA in association with increased amount of other immunoglobulin classes. No correlation of IgD and IgE values with those of the other immunoglobulins or with the activity and the duration of the disease was observed. This finding suggests that IgD and IgE regulation appears unrelated to that for IgG, IgA and IgM. Our results confirmed the data obtained by Rowe et al. (28) and Onodera et al. (25) who did not observe any modification of serum IgD in a group of subjects with rheumatoid arthritis or systemic lupus erythematosus (SLE).

The normal serum concentration of IgD and IgE in rheumatoid patients as compared to the other immunoglobulin changes remains to be explained at the present. During the past years a number of studies have been concerned with the class of immunoglobulins IgD. However, there has been little indication on the biological significance and antibody activities of these fractions compared with those of other immunoglobulins (11, 19). It has been suggested that IgD either are seldom involved in immune responses or have such a low affinity for specific antigens that antibody activity is difficult to detect (11). However, some authors found IgD antinuclear antibodies in patients with SLE (18, 27) and IgD antibodies to insulin in some diabetic patients (7), and some others observed subjects which developed IgD antibodies to milk proteins, bovine serum albumin and gamma globulin and diphtheria toxoid (11). IgD levels were not increased in some autoimmune diseases (19), but increased levels were noted in the sera from individuals with high titers of reaginic antibodies (19) and in some patients having chronic infections (28). On the contrary, it is now well known that IgE are carriers of the reaginic antibody activity (14, 15, 17). Serum concentrations of IgE were found to be significantly elevated in patients with allergic diseases (3, 8, 9, 12, 16, 21, 30, 31), but also in pathological conditions other than classical allergy (liver cirrhosis, myeloma, chronic or recurrent infections, coeliac disease) (2, 5, 10). Many factors other than the atopic state seem to be important in de-

termining serum IgE concentration, as the seasonal pollen exposure, the immunotherapy and the severity of the associated atopic disease (20). On the other hand, a significant decrease in serum IgE levels occurred with long term corticosteroid therapy (4, 20).

The nature of the factors involved in determining immunoglobulins levels is not known. Serum immunoglobulins levels represent the equilibrium between catabolism and synthesis in normal conditions, and the amount of immunoglobulins may be related to many factors, such as the environment, the genetic regulation of protein synthesis and metabolism and the antigenic stimuli (24). Particularly, some authors have stressed that the nature of the antigenic stimulation may be an important factor in determining the type of response of each immunoglobulin fraction (13). On the other hand, the absence of a parallel increase for each single component is a common finding in the group of diseases in which an increase of immunoglobulins was observed (13). Furthermore, a statistical analysis demonstrated that the amount of one class of immunoglobulin present in an individual is largely unrelated to the concentration of the other classes (1). We did not observe abnormal serum concentrations of IgD and IgE in our rheumatoid patients, neither any correlation between IgD and IgE levels and the severity of clinical symptoms. This fact and the observation that rheumatoid factors of IgM, IgG and IgA class have been well documentated in RA (26) on one hand, while no evidence was obtained up to the present time on the existence of rheumatoid factors of IgD or IgE class on the other hand, suggest that the role and the importance of IgD and IgE immunoglobulins may be excluded or considered to be negligible in hypersensitivity phenomena and in immunological response related to rheumatoid arthritis.

## Zusammenfassung

Serum-IgD- und -IgE-Immunglobuline bei primär chronischer Polyarthritis

Die Serum-IgD- und -IgE-Immunglobuline wurden bei 95 Patienten mit primär chronischer Polyarthritis, bei 5 Personen mit Sjögren-Syndrom und bei 50 gesunden Kontrollpersonen mit Hilfe einer einfachen radialen Immundiffusionstechnik sowie einer Radioimmunassay-Methode bestimmt und mit den IgG-, IgM- und IgA-Spiegeln dieser Versuchspersonen verglichen.

Bei allen 3 Versuchsgruppen ergaben sich ähnliche Werte bezüglich des Serumgehaltes an IgD- und IgE-Immunglobulinen. Eine Korrelation der IgD und IgE-Werte mit Veränderungen anderer Immunglobulinspiegel oder mit Aktivität und Dauer der rheumatischen Erkrankung wurde nicht beobachtet.

## Résumé

L'IgD et l'IgE sériques dans la polyarthrite rhumatoïde

Les immunoglobulines sériques IgD et IgE ont été déterminées par technique d'immunodiffusion radiale simple et par méthode radio-immunologique dans des

échantillons de sérums provenant de 95 malades ayant une polyarthrite rhumatoïde, de 5 sujets porteurs d'une syndrome de Sjögren et de 50 sujets témoins en bonne santé, et on les a comparées avec les taux d'IgG, d'IgM et d'IgA mesurés chez ces mêmes sujets. La teneur du sérum en IgD et en IgE a été comparable dans les cas de polyarthrite, du syndrome de Sjögren et chez les témoins. On n'a pas observé de corrélation entre les valeurs d'IgD et d'IgE et les modifications des autres immunoglobulines ou bien l'évolutivité ou la durée de la maladie rhumatoïde.

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# Exhibit F

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J Rheumatol. 1981 Mar-Apr;8(2):321-4.

# Incidence of atopy in rheumatic disease.

Peskett SA, Platts-Mills TA, Ansell BM, Stearnes GN.

Abstract

The incidence of hay fever, positive skin tests to grass pollen and specific IgG and IgE antibodies to a pollen allergen was found to be the same in a group of 40 patients with juvenile chronic arthritis as that expected in a normal population. The geometric mean total serum IgE of this group was also normal. In sera from patients with adult rheumatoid arthritis the incidence of IgG an IgE antibodies to pollen appeared to be low and the geometric mean total IgE was also low.

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# Exhibit G

*Clinical Allergy*, 1985, Volume 15, pages 547–553

# Atopy and rheumatoid arthritis

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**Summary**

The prevalence of rheumatoid arthritis (RA) was studied among 266 atopic patients attending an allergy clinic. Two patients had definite RA, a prevalence similar to that seen in the general population.

We also studied the prevalence of atopy (positive skin-prick tests) and diseases associated with atopy among forty patients with RA and forty age- and sex-matched controls. The two groups had a similar prevalence of atopy (5 RA patients, nine controls) and atopic diseases (fourteen RA patients, fourteen controls) and they did not differ with respect to blood eosinophil counts or total serum IgE. Positive RAST tests to inhaled allergens were found in three RA patients and five controls and all patients had negative RAST tests to milk and egg.

It was concluded that patients with rheumatoid arthritis have a normal prevalence of atopy and atopic diseases and we found no evidence that allergic factors contributed to the arthritis of the forty RA patients in the study.

## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown aetiology. Immune complexes and a variety of autoantibodies (including rheumatoid factors) have been detected in the circulation of patients with this disease, and it is widely believed that much of the tissue damage is caused by the type III immune response of Gell & Coombs [1]. Several abnormalities involving the type IV response (cell mediated immunity) have also been described. For example, Emery *et al.* [2] have demonstrated that delayed cutaneous hypersensitivity responses were depressed in patients with RA.

The type I response (immediate hypersensitivity) of RA patients has not been closely studied, but Peskett *et al.* [3] found that adult patients with RA had blood levels

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of IgE (total IgE and grass pollen specific IgE) which were lower than those of a control group. However, two previous studies of serum IgE in patients with RA found levels which were greater than those of a control group [4, 5].

It has also been suggested that rheumatoid arthritis might, in some cases, be an allergic disease caused by food hypersensitivity [6, 7], but larger studies involving dietary manipulation in RA patients have generally shown little or no response to 'hypoallergenic' diets [8]. Most allergists would agree that anaphylactic immediate hypersensitivity reactions sometimes cause transient joint pain and swelling [8] but these allergic joint symptoms are short-lived and do not progress to joint destruction such as occurs in rheumatoid arthritis.

For these reasons we have designed a prospective controlled study of the relationship between atopy (positive skin-prick tests to common allergens), allergic disease and rheumatoid arthritis.

## Patients and methods

### *Atopic patients*

All new patients attending Guy's Hospital allergy clinic from January 1983 to June 1984 were asked to complete a simple questionnaire seeking a personal or family history of arthritis. There were 266 atopic patients (positive skin-prick tests to one or more common allergens [9]; grass pollen, house dust mite, cat fur, dog hair and *Aspergillus fumigatus*). Whenever an atopic patient gave a history of arthritis of any sort, their hospital and general practice records were inspected for any evidence of rheumatoid arthritis or food-induced arthritis.

### *Rheumatoid arthritis patients*

Forty unselected patients with classical or definite RA [10] were studied at the rheumatology clinics and wards. The patients (eleven male and twenty-nine female) had an average age of 53 years (range 26–65) and the mean duration of disease was 14 years. The severity of their rheumatoid disease was assessed by (a) Ritchie index [11] of joint involvement (mean score 6·8); (b) duration of morning stiffness (mean 53 min); (c) erythrocyte sedimentation rate (mean 42 mm Westergren); and (d) visual analogue pain score on an unmarked 100 mm line (mean score 39 mm).

Nine of the RA patients were taking oral prednisolone. Doses ranged from 1 to 15 mg daily (median dose 4 mg). Four patients were being treated with azathioprine, two with cyclophosphamide, four with gold, nine with penicillamine and thirty-seven were taking non-steroidal anti-inflammatory drugs. No patients were taking any antihistamine medications.

### *Control patients*

For each RA patient a control of the same age (within 2 years) and sex was chosen from general medical hospital in-patients and rheumatology patients with non-inflammatory diseases. These patients (mean age 51 years) had illnesses which ranged in severity from low back pain to terminal cancer, thus they served as a diseased control group. Seventeen of these patients had inflammatory conditions, including twelve with life threatening diseases such as lung cancer or recent myocardial infarction. Seven patients were taking non-steroidal anti-inflammatory drugs and one patient was taking prednisolone.

*Questionnaires*
Each RA patient and control completed a questionnaire under the supervision of one of the authors. Any personal history of asthma, allergic rhinitis, eczema, acute urticaria or angioedema was noted and the patients were also asked whether any first degree relatives had any of these diseases. All medical problems and current medications were noted and RA patients were asked about previous adverse reactions to gold or *d*-penicillamine.

*Skin-prick tests*
The RA patients and controls were skin-prick tested using reagents purchased from Bencard Ltd, Brentford, Middlesex, U.K., (control, histamine 0·1%, mixed grass pollen, *Dermatophagoides pteronyssinus*, cat fur and *Aspergillus fumigatus*). A single batch of each solution was used throughout the study and all tests were conducted at the same time of day (14.00–18.00 hr) to allow for diurnal variation in skin test reactivity. Prick tests were performed using a precision needle with a 1 mm point (Allergy pricker, Dome Hollister Stier) [12] and the weal and flare diameters in two perpendicular axes were measured at 10 min. The mean diameter was calculated using the formula $d_1 + d_2/2$. Subjects were classed as atopic if one or more allergens gave a weal at least 2 mm greater than the control.

*Total IgE measurements*
Serum IgE levels were measured by a competitive radioimmunoassay as described by Gleich *et al.* [13]. We used a commercial radioimmunoassay kit (IgE RIA, Pharmacia GB Ltd, Milton Keynes, U.K.). Results were expressed as international units (IU) per ml (one unit = 2·4 ng IgE).

*Specific IgE measurements*
IgE antibodies to grass pollen (Timothy grass), *Dermatophagoides pteronyssinus*, cat fur, cows' milk and hen's egg were measured by the radioallergosorbent test (RAST) as previously described [14]. Allergen coated discs were prepared according to the method of Ceska *et al.* [15]. Extracts of grass pollen, *D. pteronyssinus* and cat fur were kindly donated by Beechams Ltd Surrey. These were coupled to Whatman 541 6 mm filter paper discs (20 mg extract per 2 g of discs). Milk and egg discs were prepared using neat cow's milk (15 ml per 2 g of discs) or whole hen's egg (1/10 dilution in 0·1 M $NaHCO_3$). All serum samples were assayed in duplicate at 1/10 dilution in horse serum (Sera-Lab, Guildford, U.K.). All results were read from a standard curve constructed using pooled serum from grass pollen allergic patients and expressed as scores using the Pharmacia reference system.

*Ethics*
This study was approved by the hospital ethical committee.

## Results

Of 266 atopic patients attending the allergy clinic, two had definite rheumatoid arthritis. This prevalence (0·8%) is similar to the known prevalence (1%) of RA in the general U.K. population [16]. Only one of 266 atopic patients reported joint pains which might be aggravated by food, but the clinical diagnosis in this case was osteo-arthrosis of the hips with no evidence of food allergy.

Five of forty RA patients and nine of forty control patients had one or more positive skin-prick tests to common allergens, this difference was not statistically significant (Chi squared test $P > 0{\cdot}1$). Four of the five atopic RA patients and eight of the nine atopic controls gave a history of asthma or allergic rhinitis. The mean weal and flare diameter of the positive tests was almost identical in the two groups of patients (Table 1).

Both groups had a similar prevalence of five diseases which are commonly associated with atopy, but the control patients had more asthma and allergic rhinitis, whereas the RA patients had more eczema (skin reactions to gold and *d*-penicillamine were not counted as eczema) (Table 2). A positive family history of atopic conditions was similarly common in both groups (eighteen controls, fifteen RA patients), but the RA patients were twice as likely to have a first degree relative with RA (32%) as the control patients (15%) or the atopic patients attending the allergy clinic (forty of 266 = 15%).

The average size of the weal and flare response to 0·1% histamine was closely matched in the RA patients and controls (Table 1). There was no significant correlation between histamine weal size and ESR in either RA patients or controls

**Table 1.** Skin-prick test results

| | Controls ($n=40$) | RA Patients ($n=40$) |
|---|---|---|
| Allergen skin-prick test reactions: | | |
| Number of atopic individuals (one or more positive skin-prick tests) | 9 | 5 |
| Total number of positive allergen skin tests out of 160 tests | 20 | 8 |
| Mean weal diameter (mm) of positive tests | 3·3 | 3·9 |
| Mean flare diameter (mm) of positive tests | 11·9 | 9·4 |
| Histamine skin-prick test reactions: | | |
| Mean weal diameter (mm) | 2·9 | 3·1 |
| Mean flare diameter (mm) | 10·8 | 8·7 |

**Table 2.** History of atopy-related diseases

| | Controls ($n=40$) | RA Patients ($n=40$) |
|---|---|---|
| History of any 'atopic' disease (number with positive skin-prick tests in brackets) | 14 (8) | 14 (4) |
| Asthma | 6 (4) | 3 (2) |
| Allergic rhinitis | 10 (6) | 4 (3) |
| Eczema | 2 (0) | 7 (3) |
| Acute urticaria | 5 (1) | 8 (3) |
| Angioedema | 5 (3) | 4 (0) |

(Kendall's rank correlation test) and in RA patients the histamine weal size was not significantly related to the Ritchie index, duration of morning stiffness or pain score. However, the eight patients with the most active disease all had weal and flare responses to histamine which were below the average for the group.

The mean eosinophil count was similar in the two groups (Table 3) and a similar proportion of each group (four controls, five RA patients) had an eosinophil count above 440 cells per microlitre. The mean total serum IgE had the distribution of serum IgE levels were also similar in the two groups (Table 3). A single RA patient with a serum IgE level of 1650 IU/ml produced the slightly higher mean serum IgE level in the RA group, but the median serum IgE was almost identical in the two groups. Eight RA patients had a history of adverse reactions to gold (rash or proteinuria). These patients had a mean serum IgE level and eosinophil count which did not differ from that of the other RA patients.

Three RA patients and five controls had an elevated level of specific IgE (positive RAST test) to grass pollen, house dust mite or cat fur. All of these patients also had positive skin-prick tests to these allergens. Of eight patients with one or more positive RAST tests seven had a history of asthma or allergic rhinitis (three RA patients, four controls). All RA patients and controls had negative RAST tests to milk and egg.

Only one of forty RA patients reported that her arthritis was made worse by food. She believed that citrus fruits aggravated her arthritis, but this belief was based only on her reading of reports in the lay literature on arthritis, her rheumatoid disease did not improve in any way on a diet free of these fruits.

## Discussion

This study indicates that the prevalence of rheumatoid arthritis among atopic

**Table 3.** Laboratory data

| | Controls | | RA Patients | |
|---|---|---|---|---|
| Mean ESR (mm) | 26 | | 42 | |
| Mean eosinophil count (cells per microlitre) | 180 | | 224 | |
| Mean serum IgE IU/ml (median serum IgE in brackets) | 116 | (45) | 150 | (48) |
| Number of patients with serum IgE in the following ranges (number with positive skin-prick tests in brackets) | | | | |
| 0– 50 IU/ml | 23 | (3) | 24 | (2) |
| 51– 150 IU/ml | 7 | (2) | 10 | (2) |
| 151–1000 IU/ml | 9 | (3) | 4 | (0) |
| > 1000 IU/ml | 1 | (1) | 2 | (1) |
| Number of patients with positive RAST tests to specific allergens | | | | |
| Grass pollen | 3 | | 2 | |
| *D. pteronyssinus* | 3 | | 1 | |
| Cat fur | 1 | | 0 | |
| Milk | 0 | | 0 | |
| Egg | 0 | | 0 | |

individuals is similar to that among the general population. This finding was contrary to our own clinical impression that patients with RA were rarely seen at the allergy clinic, but it also weighs against the theory that rheumatoid arthritis might be an allergic disease involving immediate hypersensitivity reactions. The possibility that RA might involve a type III or type IV immune response to foods or other allergens was not addressed in the present study, however, none of 266 atopic patients or forty RA patients had clinically evident food sensitivity.

The forty control patients had a prevalence of atopy, asthma and hay fever which was slightly (but not significantly) greater than that of the forty RA patients. However, the number of asthmatic patients among the control group (six of forty = 15%) was greater than the expected prevalence among the adult U.K. population (2–5%) [17]. This was a chance occurrence as none of the control patients had been admitted to hospital because of asthma. Furthermore, one of the original forty RA patients was found to have positive skin-prick tests but he was excluded from the study due to incomplete documentation, thus the prevalence of atopy among the original RA group was 6/40 compared with 9/40 among the control group.

The five RA patients who had positive skin-prick tests had similar weal and flare diameters to the nine atopic controls. Together with the normal eosinophil counts and serum IgE levels, this indicates that the type I immune response functions normally among RA patients. Interestingly the skin response to an inflammatory mediator (histamine) was only slightly reduced in RA patients with very active disease. The weal and flare responses in this study were smaller than those usually reported for skin-prick tests using histamine and allergens. This was the result of using a precision skin test needle which gives small but highly reproducible weal and flare responses [12]. The incidence of 'false positive' skin-prick tests in the present study was exceptionally low for this reason (only two of fourteen patients with positive skin-prick tests did not have a history of asthma or allergic rhinitis). Furthermore, the small (1 mm) point of the instrument reduces trauma to the skin and lessens the incidence of bleeding. No patients complained of discomfort from the prick tests and 480 individual prick tests produced no bleeding in any patient although many of the RA patients had extremely wasted subcutaneous tissues.

The normal skin response to histamine and allergen skin-prick tests contrasts with the impaired delayed (cell mediated) skin reactivity seen in RA patients [2]. This is probably due to the relative simplicity of the type I immune response which involves a single antigen-IgE reaction on the surface of the mast cell followed by the release of inflammatory mediators.

We did not find an elevated serum level of IgE in RA patients compared with controls. Two previous studies found that patients with RA had higher serum IgE levels than controls with osteo-arthrosis [4, 5]. However, the authors of the first study [4] used controls which were not matched for age and sex (it has subsequently been shown that serum IgE levels diminish with advancing age, especially above the age of 65 years) [18]. The high mean IgE among the RA patients was largely attributable to a small number of patients with an extremely high serum IgE. The median serum IgE was not reported in either of these studies, and as serum IgE values are not normally distributed, the median value is probably more important than the mean. Our results were similar to those of Peskett *et al.* [3] who found that total serum IgE in adults with RA was similar to (but slightly lower than) that of normal controls.

In the present study, the eight patients who had positive RAST tests were all found

to have positive skin-prick tests to the same allergens, and seven of these eight patients had a history of asthma or allergic rhinitis. No RA patients or controls gave a history of milk or egg sensitivity and all RAST tests for these allergens were negative.

We conclude that the prevalence of atopy and atopic disease among patients with rheumatoid arthritis is similar to that in the general population and the immediate hypersensitivity response as assessed by skin-prick tests, eosinophil counts, serum IgE levels and RAST tests is normal in patients with RA.

**Acknowledgments**

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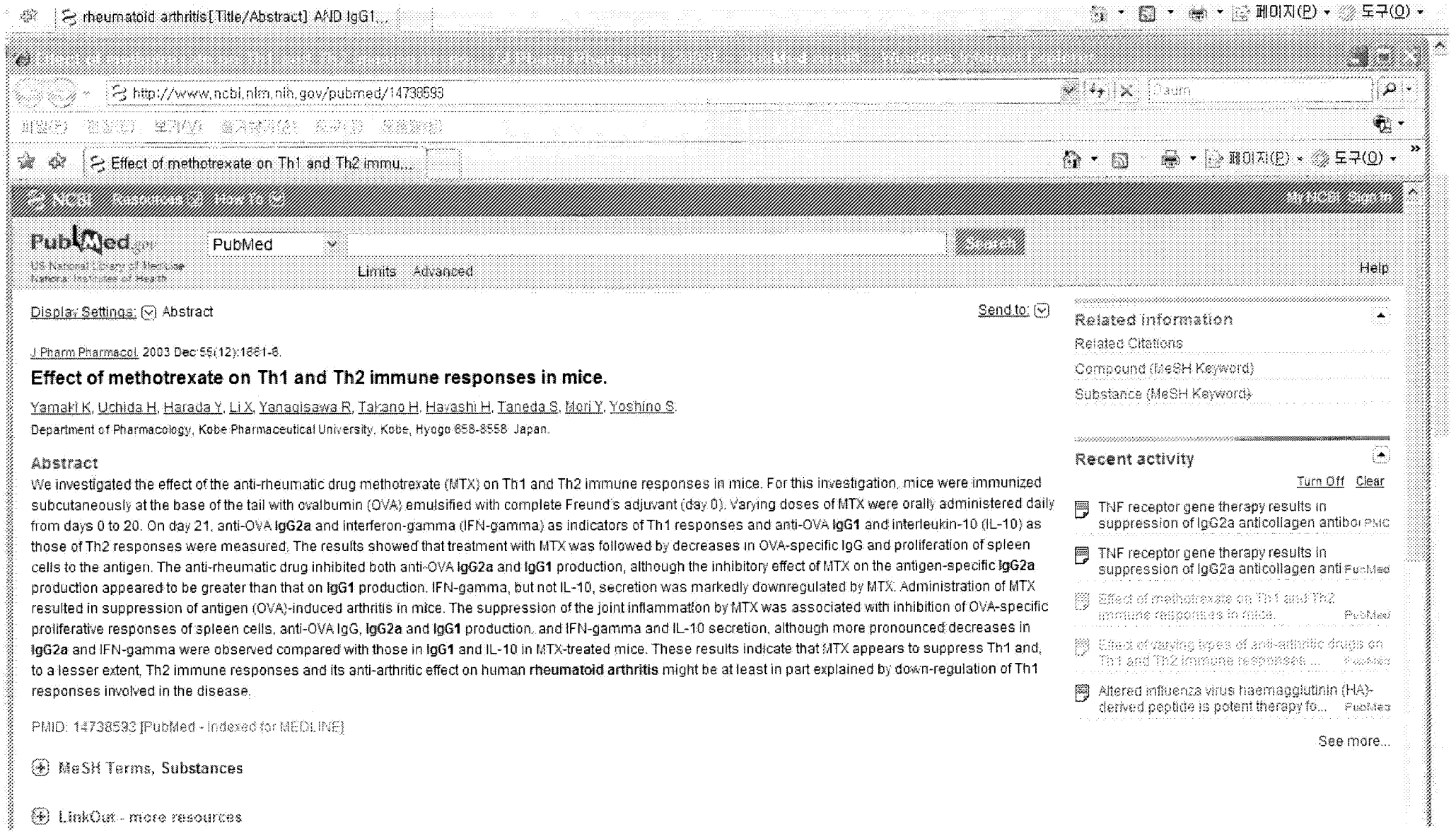
# Exhibit H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J Pharm Pharmacol. 2003 Dec;55(12):1661-6.
Effect of methotrexate on Th1 and Th2 immune responses in mice.
Yamaki K, Uchida H, Harada Y, Li X, Yanagisawa R, Takano H, Hayashi H, Taneda S, Mori Y, Yoshino S.
Department of Pharmacology, Kobe Pharmaceutical University, Kobe, Hyogo 658-8558, Japan.
Abstract
We investigated the effect of the anti-rheumatic drug methotrexate (MTX) on Th1 and Th2 immune responses in mice. For this investigation, mice were immunized subcutaneously at the base of the tail with ovalbumin (OVA) emulsified with complete Freund's adjuvant (day 0). Varying doses of MTX were orally administered daily from days 0 to 20. On day 21, anti-OVA IgG2a and interferon-gamma (IFN-gamma) as indicators of Th1 responses and anti-OVA IgG1 and interleukin-10 (IL-10) as those of Th2 responses were measured. The results showed that treatment with MTX was followed by decreases in OVA-specific IgG and proliferation of spleen cells to the antigen. The anti-rheumatic drug inhibited both anti-OVA IgG2a and IgG1 production, although the inhibitory effect of MTX on the antigen-specific IgG2a production appeared to be greater than that on IgG1 production. IFN-gamma, but not IL-10, secretion was markedly downregulated by MTX. Administration of MTX resulted in suppression of antigen (OVA)-induced arthritis in mice. The suppression of the joint inflammation by MTX was associated with inhibition of OVA-specific proliferative responses of spleen cells, anti-OVA IgG, IgG2a and IgG1 production, and IFN-gamma and IL-10 secretion, although more pronounced decreases in IgG2a and IFN-gamma were observed compared with those in IgG1 and IL-10 in MTX-treated mice. These results indicate that MTX appears to suppress Th1 and, to a lesser extent, Th2 immune responses and its anti-arthritic effect on human rheumatoid arthritis might be at least in part explained by down-regulation of Th1 responses involved in the disease.
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# Exhibit I

EXTENDED REPORT

# TNF receptor gene therapy results in suppression of IgG2a anticollagen antibody in collagen induced arthritis

**P Mukherjee, B Wu, L Mayton, S-H Kim, P D Robbins, P H Wooley**

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**Background:** Therapeutic strategies to block tumour necrosis factor α (TNFα) activity in experimental autoimmune arthritis models and rheumatoid arthritis (RA) have proved highly successful, and provide sustained beneficial effects.

**Objective:** To examine whether TNFα inhibition has immunological activity beyond the reduction of inflammation in collagen induced arthritis (CIA), an established experimental model of RA.

**Methods:** Arthritic DBA/1 mice received single periarticular injections of retroviral constructs encoding human TNF receptor (TNF-R) into the affected arthritic paw, at the onset of arthritis. Severity of arthritis, antibodies to collagen type II (CII), and extent of pathological joint damage of arthritic paws were compared between TNF-R and media treated (control) animals 3, 7, 14, 21, and 49 days after disease onset.

**Results:** Severity of CIA was significantly decreased in TNF-R treated animals compared with controls, 14–34 days after disease onset. Joint destruction was reduced in TNF-R injected joints and in the uninjected contralateral and ipsilateral paws of TNF-R treated animals. Seven days after disease onset, TNF-R treated mice had lower levels of inflammatory Th1 driven IgG2a antibodies to CII ($p<0.05$) than controls. This altered the anticollagen IgG2a:IgG1 ratio towards Th2 driven IgG1.

**Conclusions:** Local TNF-R gene therapy in CIA appears to have systemic effects on the anti-CII antibodies. The overall influence of TNF-R gene therapy is that it inhibits the progression of CIA mainly by suppressing the inflammatory Th1 response rather than by stimulating a Th2 response. Therefore, periarticular TNF-R gene therapy may have excellent therapeutic potential in RA.

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease manifested by progressive synovial joint inflammation and erosion of the subchondral bone. Interactions between synovial hyperplasia, inflammation, and an altered humoral and cellular immune system characterise the pathology of RA.[1] Collagen induced arthritis (CIA) induced in DBA/1 mice by intradermal injection of collagen type II (CII), is an animal model of RA. Histological appearance of the erosions of cartilage and bone, inflammatory and immune cytokines, genetic and clinical features associated with both RA and CIA are similar.[2] Although the aetiopathogenesis of RA is still unclear, several studies suggest that activated CD4+ T cells have an important role in initiating and perpetuating the chronic inflammation characteristic of this disease.[3–5] It has been suggested that in RA, pathophysiological processes influencing the immune response may be driven by activated Th1 cells with insufficient Th2 cell differentiation to down modulate the ongoing inflammation.[6] Skapenko *et al* showed that CD4+ memory T cells from patients with untreated RA demonstrate an intrinsic abnormality towards differentiating into specific cytokine producing effector cells that might lead to the typical Th1 dominated chronic inflammation in RA.[6] Furthermore, high levels of circulating autoantibodies to CII are always associated with CIA, and seem to be essential for development and propagation of disease. Autoantibodies to CII belonging to the IgG2 subclass have been shown to be most efficient in binding complement, another crucial factor for initiation of joint inflammation in CIA.[7 8] Also, IgG2a production is associated with a Th1 response, whereas IgG1 production is associated with a Th2 response.[9]

Tumour necrosis factor α (TNFα) is a macrophage derived cytokine with multiple inflammatory and immunoregulatory properties. Raised levels of TNFα have been found in the sera and synovial fluid of patients with RA, suggesting it has an important role in the pathogenesis of RA.[2 10] Tissue expression of TNFα and its two receptors (p55 and p75 TNF-R) is seen at several sites within the synovial membrane and the cartilage/pannus junction, indicating that a wide variety of cells may be targets for TNF activity in RA.[11–13] TNFα produced by synovial cells in RA seems to contribute to a cytokine cascade that subsequently leads to increases in interleukin (IL)1β and granulocyte macrophage-colony stimulating factor (GM-CSF) levels.[2 12–14] Studies suggest that neutralisation of TNFα down regulates the production of IL1, IL6, IL8 and GM-CSF, thereby improving the arthritic disease process[11 15–17] Therefore, TNFα represents a suitable target for intervention of the ongoing inflammatory immune process in RA.

Several different treatment immune strategies have been examined for the management of RA. Protein based anti-arthritic treatments have been shown to modulate the pathophysiology of the disease; however, they require frequent parenteral administration and are associated with several adverse side effects.[18 19] Gene therapy, requiring direct intra-articular or periarticular administration of vectors encoding for anti-inflammatory cytokines, avoids the delivery problems

**Abbreviations:** CIA, collagen induced arthritis; CII, collagen type II; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; PBS, phosphate buffered saline; RA, rheumatoid arthritis; SEM, standard error of mean; sTNF-RI, soluble tumour necrosis factor receptor I; TNFα, tumour necrosis factor α; TNF-R, tumour necrosis factor receptor

associated with protein administration.[19 20] In this study, retroviral vectors encoding for human p55 TNF-R were given periarticularly to paws of CII immunised mice on the day of arthritis onset to elucidate whether anti-inflammatory TNF-R gene therapy could ultimately influence the reactivity of autoimmune lymphocytes in CIA.

## MATERIALS AND METHODS

### Retroviral vector production

Retroviral construct—namely, MOIN-sTNF-Rc-Ig, encoded a fusion protein consisting of the extracellular domain of human 55 kDa TNFα-R covalently linked to the $C_H2$ through $C_H3$ domains of mouse IgG1 heavy chain. The soluble TNF receptor (sTNF-Rc-Ig) was amplified from sTNF-Rc-Ig plasmid using TNFsR5 and Ig3 oligomers.[21 22] The sTNF-Rc-Ig gene was inserted into the *Bam*HI site of the retroviral construct MOIN,[23] resulting in MOIN-sTNF-Rc-Ig. To produce high titre virus, the vector was transfected into Phoenix cells, a 293-based amphotropic packaging cell line, the supernatant harvested 48 hours after transfection, and titres determined by a standard plaque assay.[24]

### Induction and assessment of CIA

Female DBA/1 mice 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). Native bovine CII (provided by Dr Marie Griffiths, University of Utah) was solubilised at 2 mg/ml in 0.01 M acetic acid at 4°C overnight and emulsified with an equal volume of CFA (Difco Laboratories, Detroit, MI). Mice were given 100 µl of emulsion containing 50 µg of CII by intradermal injections at the base of their tails. Mice were weighed weekly and overall health status was noted. They were monitored daily for onset and progression of disease. Onset of CIA was determined upon observation of appearance of the first signs of definitive redness, oedema, and erythema in the metatarsal or metacarpal regions of the paws. Mice developing CIA between 20 and 55 days after immunisation were divided on the day of arthritis onset into two groups: (*a*) control (n=24) and (*b*) TNF-R treated (n=22). Arthritic paws of the mice were given periarticular injections of either 100 µl of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, NY) (group 1) or $1.6\times10^7$ pfu/ml of MOIN-sTNF-Rc-Ig in 100 µl of DMEM (group 2) at arthritis onset. Mice were assigned to one of the two groups alternately to normalise the onset date. Periarticular injections were done aseptically by guiding a Hamilton syringe with a 27½ gauge needle at a 45 degree angle into the affected joint. The retroviral vector encoding for TNF-R was injected into the joint once resistance was encountered due to the needle touching the bone in the wrist or ankle joints of the mice. In this manner both the synovial joint and the surrounding muscle cells would be transfected. Arthritic animals were clinically assessed five times a week and paw measurements were recorded three times a week for seven weeks after disease onset and at the start of treatment. An established arthritis scoring system[2] was used to evaluate disease: 0, normal appearance and flexion; 1, erythema and oedema; 2, visible joint distortion; 3, ankylosis detectable on flexion. Each limb was measured with a constant tension caliper (Dyer, Lancaster, PA) and graded, giving a maximum possible score of 12 for each mouse.

### Histology

Histological analysis was conducted to determine the extent of joint damage. Front and rear paws were removed post mortem and stored in 10% neutral buffered formalin. Paws were decalcified for 18 days in 10% formic acid, dehydrated, and embedded in paraffin blocks. Sections were cut along a longitudinal axis, mounted, and stained with haematoxylin and eosin. Specimens were cut approximately to the mid-line, and then sagittal central samples mounted for evaluation. This allowed a consistent geographic evaluation. Five to 10 samples were mounted (usually 4–6 samples for each slide), and after staining the slides were permanently bonded with coverslips. A minimum of three separate sections for each specimen was evaluated in a blinded fashion. On the front paws, wrist and metacarpal joints were scored and on the rear paws, ankle and metatarsal joints were scored. Slides were evaluated for the presence of synovitis, pannus formation, marginal erosions, architectural changes (mostly subluxation), and destruction. Synovitis was judged by thickness of the synovial membrane and scored from 0 (<3 cells thick) to 5 (>30 cells thick); pannus was scored as 0 (no pannus formation) to 5 (joint space filled by pannus and extensive pannus proliferation); erosions scored as 0 (no erosions visible) to 5 (loss of visible cartilage and major bone loss due to erosion); architectural changes scored from 0 (normal joint architecture) to 5 (complete fibrosis and collagen bridging). An overall score based on these collective points ranging from 0 (classic normal joint appearance) to 5 (destructive erosive arthritis with major bone remodelling) was then assigned to each section.

### Measurement of serum human sTNF-R levels

All mice were pre-bled before CII immunisation and then bled at onset of arthritis, and at 3, 7, 14, 21, and 49 days after disease onset and administration of treatment. Sera were separated from all samples and stored at −80°C. The levels of human sTNF-R in mouse sera were determined by a quantitative sandwich enzyme immunoassay technique using human sTNF-RI Quantikine ELISA Kit (R&D systems, Minneapolis, MN), according to the manufacturer's protocol. Briefly, 200 µl of sera and serial dilutions of standards were pipetted into wells precoated with murine monoclonal antibody against sTNF-RI and incubated for two hours at room temperature. Plates were washed three times and 200 µl sTNF-RI conjugate was added to each well and incubated for another two hours at room temperature. Unbound antibody-enzyme reagent was removed by washing the plates, and 200 µl of conjugate was added and incubated for 30 minutes. Thereafter, 50 µl of stop solution was dispensed into each well and the plates were read at 450 nm by an ultraviolet max spectrophotometer (Molecular Devices, CA). Negative pre-bleed control sera and human sera with a 1000-fold dilution were titred on each plate to ensure uniformity of the assay.

### Measurement of serum anti-type II collagen antibody levels

All mice were pre-bled before CII immunisation and then bled at onset of arthritis, and at 3, 7, 14, 21, and 49 days after disease onset. Sera were separated from all samples and stored at −80°C, and the levels of serum antibovine CII antibodies were determined by enzyme linked immunosorbent assays (ELISAs) as described previously.[25] Briefly, ELISA plates (Nunc-Immuno plates, Denmark) were coated with 100 µl of coating buffer (0.4 M phosphate buffer pH 7.6) containing 3 µg of bovine collagen II, at 4°C overnight. Plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (Sigma, St Louis, MO), and non-specific binding was blocked by PBS containing 5% non-fat milk overnight at 4°C. One hundred microlitres of mouse sera diluted at 1/1000 in 5% milk/PBS was added to each well, except blank wells, and incubated overnight at 4°C. Subsequently, the plates were washed six times in PBS containing 0.05% Tween 20 and incubated with alkaline phosphatase conjugated goat antimouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL) at 37°C for one hour. In assays to determine the isotype of the bound antibody, alkaline phosphatase conjugated goat antimouse antibodies specific for IgG, IgG1, or IgG2a were used. Plates were washed six times again and developed for 5–20 minutes in the dark by the addition of

*p*-nitrophenylphosphate (Sigma, St Louis, MO) as a chromatogen substrate. The resulting optical density was measured at 405 nm by an ultraviolet max spectrophotometer (Molecular Devices, CA). Negative pre-bleed control sera and a standard mouse anti-CII antiserum were titred onto each plate to ensure uniformity of the assay. Antibody binding was expressed as $OD_{405}$ units.

### Analysis of local TNFα expression in mice paws

Front and rear paws were removed post mortem at 3, 7, 14, 21, and 49 days after disease onset and treatment. Skin was removed and paws cut into 3–4 pieces and homogenised in lysis buffer using a Polytron tissue homogeniser (Kinematica Inc, Switzerland). Insoluble debris was removed from homogenised tissue by centrifugation at 12 000 *g* at 4°C for 15 minutes. Murine TNFα was measured in tissue lysates by sandwich ELISA. In brief, 96 well ELISA plates (Nunc-Immuno plates, Denmark) were coated overnight with 50 µl of purified rat antimouse TNFα antibody (Pharmingen, San Diego, CA) at 4°C. Plates were washed with PBS/0.05% Tween 20 (Sigma, St Louis, MO), and non-specific protein binding sites were blocked with 10% fetal calf serum in PBS for six hours at room temperature. Samples and standards were added and kept overnight at 4°C. Plates were washed again to remove unbound proteins, and 50 µl biotin conjugated secondary rat antimouse TNFα antibody (Pharmingen, San Diego, CA) was added to each well. After a one hour incubation at 37°C, plates were washed and again incubated at room temperature with an avidin-alkaline phosphatase conjugate (Pharmingen, San Diego, CA). After washing, *p*-nitrophenylphosphate substrate (Sigma, St Louis, MO) was added and plates were developed in the dark for 15–20 minutes. The resulting optical density was measured at 405 nm by an ultraviolet max spectrophotometer (Molecular Devices, CA).

### Statistical analysis

Data were analysed using the SPSS-PC statistical software (SPSS, Chicago, IL). Group comparisons between TNF-R treated and control mice were performed by the two tailed independent *t* test. Comparisons of more than two means were conducted using the one way analysis of variance, and $p<0.05$ was considered to be significant.

## RESULTS

### Effect of retroviral vector mediated periarticular delivery of TNF-R on collagen arthritis

Onset of arthritic disease was defined as the appearance of definitive signs of oedema and erythema in the paw. Onset of arthritis occurred in a single joint in all animals, and subsequently progressed to other joints. The joint exhibiting the first signs of clinical arthritis received a single periarticular injection of 100 µl of MOIN-sTNF-Rc-Ig containing a viral titre of $1.6\times10^7$ pfu/ml, at arthritis onset (day 1), while control animals received equal volumes of media into the first affected paw. Progression of arthritis was evaluated until 49 days after onset of arthritis, and the number of paws affected and the mean clinical scores were recorded. MOIN-sTNF-Rc-Ig treatment led to a significant reduction in the number of arthritic limbs affected between 15 and 25 days after disease onset ($p<0.05$) (fig 1A). Mean paw score was significantly reduced ($p<0.05$) in MOIN-sTNF-Rc-Ig treated animals compared with the control animals between 14 and 34 days after disease onset (fig 1B). Overall, these results demonstrate that local delivery of MOIN-sTNF-Rc-Ig significantly ameliorates the arthritic disease process up to 34 days after treatment. These experiments were repeated twice, and consistent results obtained.

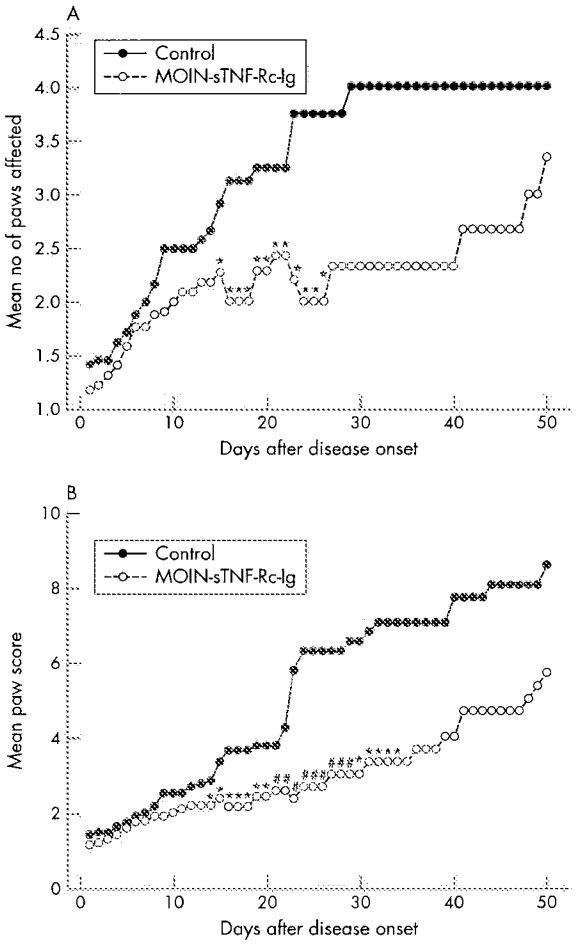


**Figure 1** Effect of a single periarticular delivery of retrovirus mediated MOIN-sTNF-Rc-Ig on the clinical severity of CIA. At arthritis onset 100 µl of media alone (n=24) or $1.6\times10^7$ pfu/ml of MOIN-sTNF-Rc-Ig in 100 µl of media (n=22) was injected periarticularly into the affected arthritic paw. Disease activity represented by (A) the mean number of paws affected and (B) the mean paw score was visually scored up to 49 days after disease onset. Data are expressed as mean for each group. SEM <5% of mean (not shown). Statistically significant differences between the two groups is indicated: *$p<0.05$; #$p<0.02$). The figure shows representative results from two different individual experiments, which gave similar results.

### Histological assessment of paws of control and MOIN-sTNF-Rc-Ig treated animals

Examination of the joint pathology demonstrated that the degree of synovitis, erosion, architectural changes of the bone, and overall joint destruction were significantly reduced in the arthritic paws of the MOIN-sTNF-Rc-Ig treated mice compared with the control animals (figs 2 and 3). The MOIN-sTNF-Rc-Ig injected paws showed a considerable reduction ($p<0.05$) in disease pathology compared with media injected control paws. The histological scores also revealed a significant reduction in the degree of joint destruction in the contralateral ($p<0.05$) and ipsilateral ($p<0.01$) paws compared with the control paws. These findings suggest that local periarticular delivery of MOIN-sTNF-Rc-Ig significantly reduces the ongoing disease process in the affected paw, and has marked systemic effects that block the progression of the inflammatory process to the uninjected joints, thereby preventing end stage destructive bone damage in these arthritic joints. This was consistent with levels of IL1β and

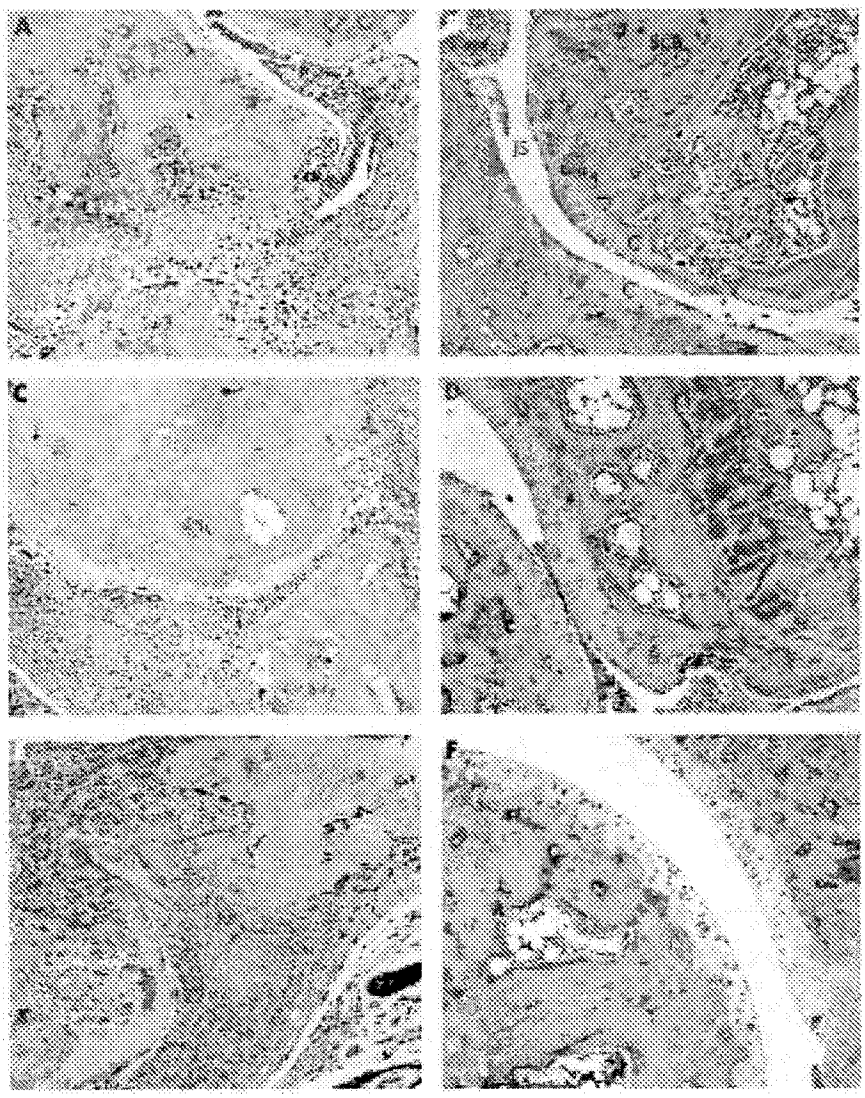


**Figure 2** Histopathology of injected, contralateral, and ipsilateral joints. Histological evaluation of control injected (A), contralateral (C), and ipsilateral (E) and MOIN-sTNF-Rc-Ig injected (B), contralateral (D), and ipsilateral (F) arthritic joints were conducted at 49 days after disease onset and at the start of treatment. Media injected control joints show severe synovial inflammation with clear pannus attachment and erosions extending into the subchondral bone, indicating destructive erosive disease. Similar pathological changes are seen in the control arthritic contralateral and ipsilateral joints. Injected, contralateral, and ipsilateral arthritic joints of MOIN-sTNF-Rc-Ig treated mice show a lesser degree of both synovial inflammation and destructive disease, indicating a relatively normal joint architecture. Haematoxylin and eosin staining (magnification ×100) used for all sections. JS, joint space; SCB, subchondral bone; CPJ, cartilage/pannus junction; Syn, synovium; P, pannus.

TNFα mRNA measured in the paws. The contralateral and ipsilateral paws of the MOIN-sTNF-Rc-Ig treated animals had lower levels of both these cytokines than the injected and the similar paws of the control animals (data not shown).

### Effect of retroviral gene therapy delivering TNF-R on anti-CII antibody levels

Serum samples were obtained from both control and treated animals at 3, 7, 14, 21, and 49 days after disease onset. The effect of MOIN-sTNF-Rc-Ig treatment on the humoral response to CII at these different times was determined by assaying the sera for antibodies to bovine CII (fig 4). Total immunoglobulin levels were lower in MOIN-sTNF-Rc-Ig treated animals than in controls ($p<0.05$) three weeks after onset of arthritis and administration of treatment (fig 4A). Subsequently, the isotype of the anti-CII antibody response was assessed at different times. Lower anti-CII IgG levels were seen in MOIN-sTNF-Rc-Ig treated animals than in controls ($p<0.05$) seven days after treatment (fig 4B). No significant differences were seen in the anti-CII IgG1 levels between the two groups (fig 4C); however, the anti-CII IgG2a titres were significantly lower ($p<0.05$) in sera from MOIN-sTNF-Rc-Ig treated mice than in controls seven days after arthritis onset (fig 4D). At that time, because anti-CII IgG1 levels were similar in the two groups but anti-CII IgG2a titres were markedly decreased in the TNF-R treated mice, a significant shift ($p<0.005$) in the anti-CII IgG2a:IgG1 ratio towards IgG1 was seen (fig 5). These observations suggested that although periarticular anti-inflammatory TNF-R gene therapy does not cause an isotype switch of the anti-CII autoantibody, it leads to an alteration in the anti-CII IgG2a:IgG1 ratio towards IgG1 by predominantly down regulating the Th1 mediated IgG2a response rather than up regulating the Th2 driven IgG1 response. The suppressive effect on IgG2a antibodies is consistent with an influence on the Th1 cells rather than Th2 cells. We have demonstrated that periarticular administration

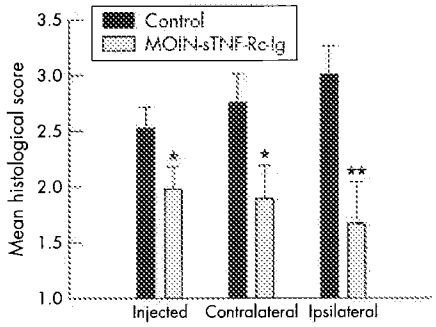


**Figure 3** Histological scoring of arthritic joints. Arthritic joints were analysed by histology using haematoxylin and eosin staining for extent of joint damage and assigned a histological score (as described in "Materials and methods"). Each column represents the mean scores (SEM) for the total number of mice in each group. *p < 0.05; **p<0.01.

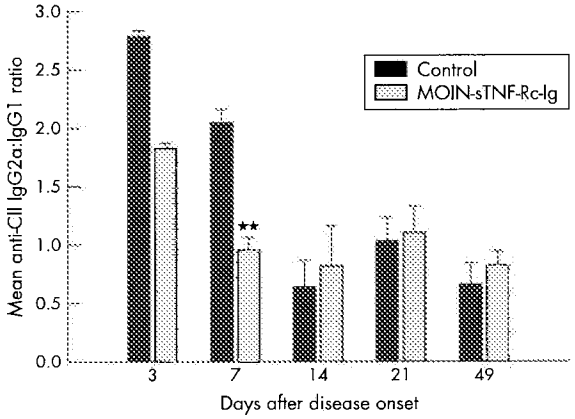


**Figure 5** Effect of a single periarticular delivery of MOIN-sTNF-Rc-Ig on the antibovine CII IgG2a:IgG1 ratio. Titres of anti-bovine CII IgG1 and IgG2a were measured by ELISA. The anti-CII IgG2a:IgG1 ratio 3, 7, 14, 21, and 49 days after disease onset is represented. Each column represents the mean ratios (SEM) for the total number of mice in each group for each time. n = 5 for both the media and MOIN-sTNF-Rc-Ig treated mice. **p<0.005.

of retroviral construct MOIN encoding for marker gene *Lac*Z into the arthritic paw had no effect on the clinical disease in CIA or the anti-CII antibody profile, suggesting that the above observations were a result of TNF-R and not an effect of the retroviral vectors (data not shown)

### Measurement of local TNFα expression in control and MOIN-sTNF-Rc-Ig treated animals

Local expression of TNFα in the joints of MOIN-sTNF-Rc-Ig treated and control animals was measured by ELISA at 3, 7, 14, 21, and 49 days after treatment and disease onset. It was noted that the TNFα levels in the MOIN-sTNF-Rc-Ig injected paws were slightly higher than in media injected control joints, although these differences were not statistically significant (fig 6A). Paradoxically, it was observed that local TNFα levels in the uninjected arthritic ipsilateral joints of the MOIN-sTNF-Rc-Ig were lower than the ipsilateral joints of the control animals at all times, achieving statistically significant values 14 days after disease onset (fig 6B). Similar results were also seen in the arthritic contralateral joints (data not shown). Consequently, when the local TNFα expression in all the

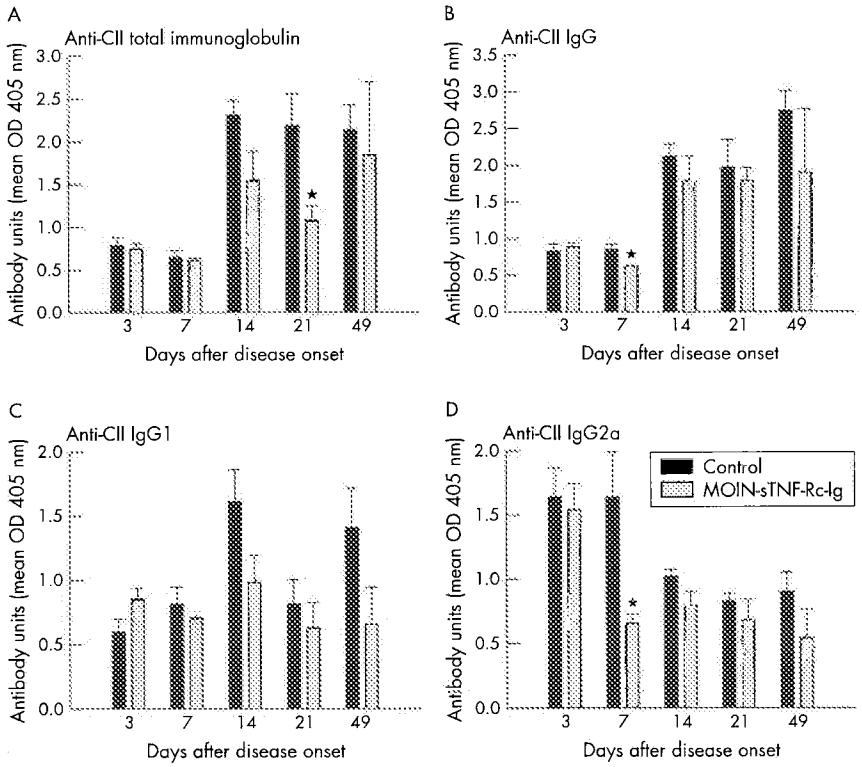


**Figure 4** Effect of periarticular delivery of MOIN-sTNF-Rc-Ig on the antibody profile to bovine CII. At arthritis onset mice received single periarticular injections of MOIN-sTNF-Rc-Ig or media into the affected arthritic paw. Serum samples were collected 3, 7, 14, 21, and 49 days after arthritis onset. Titres of antibodies to bovine CII were measured by ELISA. Each column represents the anti-bovine CII (A) immunoglobulin; (B) IgG; (C) IgG1; and (D) IgG2a levels in the treated or control sera at these times. Values are means (SEM) for the total number of mice in each group for each time. n=5 for both the media and MOIN-sTNF-Rc-Ig treated mice. *p<0.05.

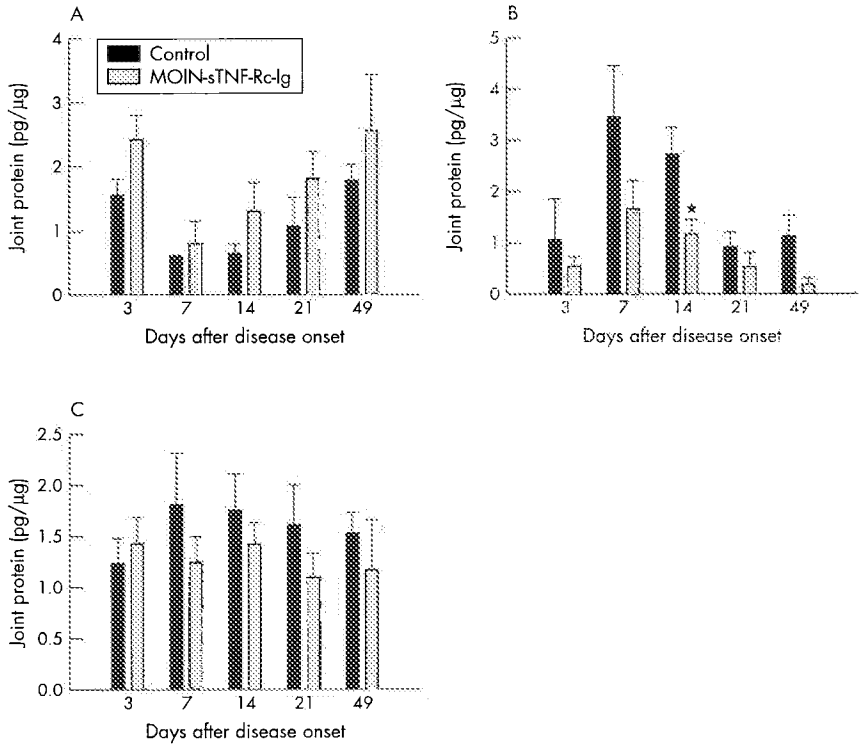


**Figure 6** Effect of a single periarticular delivery of MOIN-sTNF-Rc-Ig on the local TNFα levels. Local TNFα levels present in tissue lysates obtained from (A) injected (B) ipsilateral paws of control and MOIN-sTNF-Rc-Ig treated mice, harvested at 3, 7, 14, 21, and 49 days after disease onset were measured by ELISA. Contralateral joints showed results very similar to the ipsilateral joints. The cumulative levels of local TNFα expression in the injected, ipsilateral, and contralateral joints of the treated and control animals are represented in (C). Each column represents the mean (SEM) TNFα levels for the total number of mice in each group for each time. n = 5 for both the media and MOIN-sTNF-Rc-Ig treated mice. *p<0.05.

injected, contralateral, and ipsilateral joints was cumulatively analysed, an overall reduction in the TNFα was observed in MOIN-sTNF-Rc-Ig treated animals compared with controls from 7 to 49 days after disease onset (fig 6C). These results showed that MOIN-sTNF-Rc-Ig was not completely successful in lowering the TNFα levels in the first joints to be affected. This can be explained by the markedly raised levels of proinflammatory cytokines[26 27] associated with the onset of clinical disease and inflammation in CIA. It appears that our therapeutic treatment given at the onset of arthritis cannot effectively lower the already raised levels of TNFα present in the joint at disease onset but can significantly reduce TNFα levels in uninjected joints as they become affected, leading to overall disease amelioration.

Because local MOIN-sTNF-Rc-Ig treatment had beneficial systemic effects we assayed the expression of human TNF-R levels in sera over the time course of the experiment. Serum human sTNF-R levels from both control and MOIN-sTNF-Rc-Ig treated mice obtained at different times were measured by ELISA. Three days after disease onset and MOIN-sTNF-Rc-Ig administration, the human sTNF-R level in the circulation was 16.7 pg/ml. This value fell to 12.7 pg/ml seven days after disease onset and treatment and thereafter was undetectable at 14, 21, and 49 days after onset of disease. Therefore, the beneficial systemic effects of periarticular TNF-R gene therapy on the anti-CII antibodies and local TNFα expression in uninjected paws correlated well with the ability to measure circulating levels of human TNF-R. However, local expression of human TNF-R in the joints of the MOIN-sTNF-Rc-Ig could not be detected, possibly owing to either the rapid diffusion of the protein or the dilution effects during paw protein extraction. Therefore, although local expression of human TNF-R was below the level of detection in our assay system, periarticular TNF-R therapy resulted in a generalised reduction in the local TNFα levels in the treated animals.

## DISCUSSION

CIA and RA have several common immunological and pathological features,[28] including the involvement of inflammatory cytokines in the arthritic aetiology. IL1 levels are increased in mice with CIA. Whereas the administration of recombinant IL1 to mice with CIA aggravates the disease process,[2 29] treatment with IL1 receptor antagonist protein inhibits both RA and CIA,[30–32] thus implicating IL1 as one of the mediators of the inflammatory process in this disease.[33] IL2 and IL6 are also associated with the immunopathogenesis of CIA.[34 35] These results provide compelling evidence that many cytokines participate in an immune network regulating the pathogenesis of RA. The onset of clinical symptoms and inflammation in CIA is highly dependent upon a predominantly Th1 response, characterised by the presence of anticollagen IgG2a antibodies and the proinflammatory cytokines TNFα and interferon γ.[26 27] TNFα participates in the cytokine cascade by up regulating IL1β and GM-CSF levels, leading to synovial inflammation and joint erosion.[2 15 17 20] Anti-TNFα monoclonal antibody therapy has been shown to ameliorate disease significantly both in clinical trials with patients having longstanding RA and in experimental arthritis.[36] Our results support previous findings[2 37–39] and indicated that TNF-R delivered locally at the arthritic paw using a retroviral vector inhibited the disease by reducing the number of paws affected and the severity of arthritis. Histology revealed that while TNF-R injected paws showed significant local effects, including reduced bone damage and a marked improvement in disease, more pronounced protective effects occurred in the uninjected paws. This suggests that the therapeutic treatment given at the onset of

disease may not completely down regulate the raised TNFα levels and inflammatory process already initiated in the affected joint, but may exert a systemic effect that reduces TNFα secretion and the development of the inflammatory process in other joints, as they become affected. This hypothesis is supported by the lower levels of local TNFα expression found in uninjected arthritic paws compared with the injected arthritic paws.

The systemic effects of the local retrovirus mediated TNF-R gene therapy correlate well with the circulating serum human TNF-R levels in treated mice. The observation of reduced joint erosions and bone destruction in treated animals is new, because Joosten *et al* reported that TNFα blockade by intraperitoneal injections of TNF binding protein only improved joint inflammation and had little effect on cartilage and bone destruction,[40] whereas IL1 inhibition had a greater effect on reducing bone destruction than TNFα blockade. Possibly, the periarticular route of delivery of the retroviral vectors in our study was effective in concentrating TNF-R within the joint and thereby reducing bone destruction. Because TNFα blockade leads to down regulation of IL1,[15–17] which in turn may inhibit cartilage erosion and bone resorption, the successful outcome of TNF-R gene therapy seen in our study might well be expected.

We also demonstrated that periarticular delivery of TNF-R had a marked effect on the humoral response to bovine CII, with a reduction of anti-CII total IgG levels, and moreover a decrease in the anticollagen IgG2a antibody. Anti-CII IgG2a plays a crucial part in the immunopathogenesis of CIA.[7 8] Further, deposition of IgG antibodies and complement fixation occurs immediately before the development of overt arthritis.[41] In our study, reduction in levels of anti-CII IgG2a led to an alteration in the ratio of IgG2a and IgG1 in favour of Th2 driven anti-CII IgG1 at seven days after disease onset. Various studies have reported an activated Th1 response in the absence of an adequate counterbalancing Th2 response before the clinical onset of CIA[42 43] and that a switch from a Th1 to Th2 profile leads to a clinical improvement in both CIA and RA.[33 37 44] We show that local TNF-R gene therapy significantly lowered the Th1 mediated IgG2a antibody response early in the disease, but did not influence the Th2 driven IgG1 antibody levels. Because the anti-CII IgG1 levels were not affected and only anti-CII IgG2a titres were reduced, isotype switching of the autoantibody was not apparent. Instead, a shift towards IgG1 rather than a switch in the anti-CII IgG2a:IgG1 ratio was observed. The alleviation of the clinical signs caused by TNF-R therapy up to 34 days after disease onset can be attributed mainly to the down regulation of the Th1 mediated inflammatory response rather than to the up regulation of a Th2 driven IgG1 response, in agreement with the finding of Horsfall *et al* that continuous administration of IL4 improved CIA by suppressing the Th1 response without any effect on Th2 responses.[27]

In this study the reduction in anti-CII IgG2a levels was not sustained beyond seven days and, also, clinical improvement started diminishing 34 days after treatment. This is consistent with our finding that the human TNF-R levels were undetectable in the circulation eight days after disease onset and MOIN-sTNF-Rc-Ig treatment. This might be an effect of the single administration of MOIN-sTNF-Rc-Ig given at disease onset in our study. In unpublished studies we have shown that retroviral vectors encoding a marker *Lac*Z gene, injected periarticularly into the affected joints at arthritis onset showed good *Lac*Z transduction into the injected joint, but had no effect on the clinical disease or on the anti-CII antibody profile in CIA. Thus, these findings indicate that the outcome of MOIN-sTNF-Rc-Ig treatment on CIA is an effect of the anti-inflammatory TNF-R rather than the retroviral vector. A previous study showed paradoxical effects after adenovirus mediated delivery of TNF-R, with amelioration of arthritis 10 days after treatment followed by a rebound in disease despite the presence of bioactive TNF-R fusion protein.[36] This discrepancy with our findings may be due to different vectors and routes of delivery of the anti-inflammatory cytokines. The retroviral vectors in our study were given periarticularly, whereas the previous study used an intravenous route. Additionally, the delayed rebound inflammation observed in the latter study might possibly be attributed to the immunogenic effects associated with the use of adenoviruses.[45 46] A previous report indicated that periarticular delivery of viral IL10 by adenoviral vectors inhibited CIA development in both the injected and uninjected paws, and significantly reduced the antibodies to autologous CII while having no effect on the circulating antibodies to heterologous CII.[20] These differences suggest that cytokines other than TNF are critical to the development of autoantibodies in this model.

In conclusion, these findings suggest that local TNF-R treatment has systemic effects that inhibit the production of the proinflammatory cytokine TNFα in the uninjected arthritic joints and suppress Th1 cells without up regulating the Th2 cells, eventually leading to an overall improvement in CIA. Future studies will be done to evaluate whether repeated periarticular administrations of retrovirus mediated TNF-R might have a more sustained effect on the humoral response and clinical progression of disease in CIA. Thus, retroviral vector mediated periarticular delivery of the anti-inflammatory cytokine TNF-R may be a suitable strategy for alleviating disease in patients with RA.

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# Exhibit J

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# The balance of Th1/Th2 cytokines in rheumatoid arthritis

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It has been suggested that rheumatoid inflammation is mediated by activated pro-inflammatory T helper type I cells. In contrast, immunomodulatory T helper type 2 cells and their cytokines, in particular interleukin-4, are rarely found. This chapter reviews the concept of the Th1/Th2 dichotomy and summarizes the functions of the signature cytokines of the T helper subsets. We discuss current knowledge of the immunopathogenesis of rheumatoid arthritis and its related animal model, collagen induced arthritis, with regard to the Th1/Th2 paradigm. The accumulating evidence for a T helper type 1 driven inflammation and the implications for future therapy are delineated.

**Key words:** rheumatoid arthritis; Th1/Th2; autoimmunity; cytokines; pathogenesis; treatment.

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that is characterized by persistent intense immunological activity, local destruction of bone and cartilage, and a variety of systemic manifestations.[1] Although the present understanding of the pathogenesis of the disease is still incomplete, there is convincing evidence to support the conclusion, that $CD4^+$ T cells play a central role in initiating and perpetuating the chronic autoimmune response characteristic of rheumatoid inflammation.[1] $CD4^+$ T cells are activated by an antigen, i.e. peptide, recognized specifically by their T cell receptor (TCR) if presented in the context of a specific MHC class II molecule on the surface of an antigen-presenting cell. Once activated, $CD4^+$ T cells become the central regulators of specific immune responses and determine to a large extent the outcome of immune reactions by activating different effector functions of the immune system. It is of no surprise, therefore, that activated $CD4^+$ T cells can be found in the inflammatory infiltrates of the rheumatoid synovium and that T cell-directed therapies have provided some clinical benefit in RA. The most compelling data, however, implying a central role for $CD4^+$ T cells in propagating rheumatoid

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inflammation remain the association of aggressive forms of the disease with particular MHC class II alleles, such as subtypes of HLA-DR4, that contain similar amino acid motifs in the third hypervariable region of the DRβ-chain.[2] Although the exact meaning of this association has not been resolved, all interpretations imply that $CD4^+$ T cells orchestrate the local inflammation and cellular infiltration, following which a large number of subsequent inflammatory events are unleashed. Moreover, recent evidence suggests that the role of T cells in RA seems not to be restricted to the synovium, and their pathogenic role may extend beyond antigen recognition in the joint.

Great efforts have been made in recent years to delineate more precisely the nature of the $CD4^+$ T cells involved in rheumatoid inflammation. Whereas the specific antigen(s) eliciting the detrimental autoimmune response is still unknown, much progress has been made in defining the phenotype and function of $CD4^+$ T cells in RA. In 1986, Mosmann and colleagues discovered that repeated antigen-specific stimulation of murine $CD4^+$ T helper (Th) cells in vitro resulted in the development of restricted and stereotyped patterns of cytokine secretion profiles in the generated T cell populations.[3] Subsequently, it was observed that many experimental models of autoimmune diseases in animals are characterized by the dominant activation of a particular Th subtype expressing pro-inflammatory cytokines (Th1 cells).[4] These findings have fostered a huge volume of work that has been devoted to categorizing the pathogenesis of inflammatory arthritis in humans with respect to the polarized Th effector subsets. In this chapter we discuss the concept of the Th1/Th2 dichotomy, briefly summarize the functions of the key cytokines of the Th subsets and review the current knowledge of the immunopathogenesis of RA and its related animal model, collagen-induced arthritis (CIA), with regard to the Th1/Th2 paradigm. Although the attempts to dichotomize complex diseases such as RA in terms of just Th1 or Th2 may be an oversimplification, the concept not only clearly allows a better understanding of the mechanisms involved in the pathogenesis of the disease but also provides the basis for the development of novel strategies for the treatment of RA.

## THE TH1/TH2 DICHOTOMY

Based on their distinctive cytokine secretion pattern and effector functions, $CD4^+$ T cells can be divided into at least three different subsets. Th1 cells develop preferentially during infections with intracellular bacteria. Upon activation, Th1 cells secrete the pro-inflammatory cytokines interleukin (IL)-2, interferon (IFN)-γ and lymphotoxin (LT, tumour necrosis factor (TNF)-β). They activate macrophages to produce reactive oxygen intermediates and nitric oxide (NO), stimulate their phagocytic functions and enhance their ability for antigen presentation by upregulation of MHC class II molecules. Moreover, Th1 cells promote the induction of complement-fixing, opsonizing antibodies and of antibodies involved in antibody-dependent cell cytotoxicity, for example, IgG1 in humans and IgG2a in mice. Consequently, Th1 cells are involved in cell mediated immunity. Immune responses driven by Th1 cells are exemplified by the delayed-type hypersensitivity reaction.[3,4]

Th2 cells predominate after infestations with gastrointestinal nematodes and helminths. They produce the anti-inflammatory cytokines IL-4 and IL-5 and provide potent help for B cell activation and immunoglobulin class switching to IgE and subtypes of IgG that do not fix complement, for example, IgG2 in humans and IgG1 in the mouse. Th2 cells mediate allergic immune responses and have been associated with down-regulation of macrophage activation, which is mediated largely by the anti-inflammatory

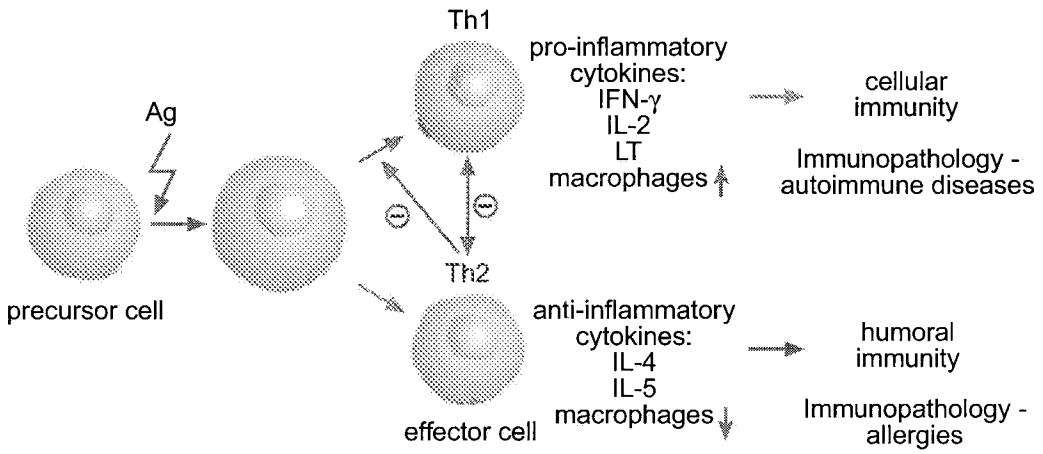


**Figure 1.** Differentiation of T helper cells into Th1 or Th2 effector cells. Upon activation with specific antigen (Ag), $CD4^+$ Th cells can differentiate into either the Th1 or Th2 subset, depending on the milieu and the signals they receive during priming. Th1 effectors are pro-inflammatory and promote cellular immunity; they are involved in autoimmune diseases. Th2 effectors are anti-inflammatory; they promote humoral immunity and mediate allergic immune responses. Th1 and Th2 cells antagonize each other by blocking each other's effector functions. Th2 cells also block the development of Th1 effectors.

effects of IL-4 [3,4] (Figure 1). Th2 cells can also secrete IL-6, IL-10 and IL-13; however, in contrast to the situation in mice, these cytokines in humans are not confined to the Th2 subset but can also be produced by Th1 cells.[4]

Recent evidence has suggested that Th1 and Th2 cell populations differ not only in the cytokines they produce but also in their migratory capacity.[5] A large number of studies have demonstrated a preferential expression of chemokine receptors on activated effector cells from one or the other subset in in vitro cell culture and in animal experiments. However, analysis of $CD4^+$ memory T cells from the rheumatoid synovium at the single-cell level failed to show a clear association of chemokine receptor expression and cytokine production, supporting the conclusion that specific surface markers for a given population might probably not exist in an in vivo situation in humans.[6]

It has been shown that the different functional Th subsets do not derive from different pre-committed lineages but may rather develop from the same uncommitted precursor cell under the influence of environmental and genetic factors.[7] Cytokines are the most important regulators of Th subset differentiation. Whereas IL-2 is required for the differentiation of naive cells into either Th subset without imposing a functional bias, priming of naive $CD4^+$ T cells in the presence of IL-4 induces differentiation of Th2 effector cells. In contrast, Th1 cell development occurs in the absence of IL-4 and is greatly enhanced by IL-12.[4] Other factors that control Th subset polarization include the nature and intensity of co-stimulatory signals, in particular via CD28 and OX40, the intensity of TCR ligation during priming, the type of antigen-presenting cells, the MHC class II genotype, minor histocompatibility complex genes, and corticosteroids or endogenous hormones.[4]

Importantly, Th1 and Th2 cells antagonize each other by blocking the generation of the antipodic cell type and by blocking each other's effector functions. For instance, the generation of Th1 cells can be effectively blocked by high concentrations of IL-4, even in the presence of IL-12.[8] At the level of effector functions, IL-4 antagonizes much of the pro-inflammatory effect of IFN-γ and inhibits the proliferation of Th1 cells. Conversely, IFN-γ secreted by Th1 cells blocks the proliferation of Th2 cells (Figure 1).

Differentiation of the appropriate Th subset is of crucial importance to the host in mounting protective immunity against exogenous microorganisms. However, it has become apparent in recent years that immune responses driven preferentially by activated Th subsets are also involved in the development of pathological immune disorders. Whereas atopic disease result from Th2-dominated responses to environmental allergens, Th1-mediated immunity is involved in the generation of several organ-specific experimental autoimmune diseases in animals, such as experimental allergic encephalomyelitis, insulin-dependent diabetes mellitus, or collagen-induced arthritis (CIA), an animal model for RA.[4] Moreover, evidence is accumulating to suggest that human autoimmune diseases might also be driven by a preferentially activated Th subset. The arguments supporting the conclusion of a Th1 drive in RA are presented in this chapter in detail.

A third subset of T cells, designated T regulatory (Tr) cells, has recently been described, first in mice and subsequently in humans. These T cells produce large amounts of IL-10 but no IL-4. It is speculated that Tr cells might play a significant role in maintaining peripheral tolerance.[9] However, the precise function of these cells in immunohomeostasis and, moreover, in autoimmunity, has only begun to be addressed.

## IL-4

IL-4 was discovered as a T cell product distinct from IL-2 that could stimulate anti-IgM-treated B cells to proliferate and to differentiate into IgG-secreting plasma cells. IL-4 is a 20 kDa secreted glycoprotein that elicits a huge number of diverse biological responses in many different types of cell. IL-4 is produced by activated T cells, mast cells, NK1.1 T cells, basophils and eosinophils. Its main functions on the immune system are its dominant ability to direct T cell differentiation into the Th2 subset and its role in mediating immunoglobulin class switching to the IgG1 and IgE isotypes in mice and to the IgG4 and IgE isotypes in humans. IL-4 is the signature cytokine of Th2 cells and suppresses Th1 development while promoting Th2 generation. IL-4 is an important growth factor for T and B cells and increases the survival of cultured human lymphocytes. Overproduction of IL-4 has been associated with elevated IgE production and allergic diseases in vivo. Of importance in regulating immune responses is its ability to downregulate the activation and the inflammatory functions of monocytes and macrophages. IL-4 increases the expression of MHC class II molecules and of several cytokine inhibitors such as IL-1 receptor antagonist (IL-1Ra), soluble IL-1 receptor type II and TNF receptors while downregulating the production of the pro-inflammatory cytokines IL-1, TNF-$\alpha$, IL-6, IL-8 and IL-12. The ability of Th2 effectors to control Th1-mediated inflammatory responses has been attributed largely to these anti-inflammatory effects of IL-4. Consequently, IL-4 has been used in vivo as a treatment for a great number of experimental autoimmune diseases in animals. It is, to date, the most successful means for ameliorating autoimmune disorders that are caused by activated Th1 cells. For example, IL-4 improves experimental allergic encephalomyelitis, delays the onset and diminishes clinical symptoms of CIA and prevents joint damage and bone erosion in this experimental autoimmune disease.[10] In vitro, IL-4 suppresses metalloproteinase production and stimulates tissue inhibitor of metalloproteinases (TIMP)-1 production in human mononuclear phagocytes and cartilage explants, indicating a protective effect of IL-4 towards extracellular matrix degradation. Furthermore, IL-4 inhibits bone resorption through an effect on both osteoclast activity and survival[11] and reduces the spontaneous secretion of pro-inflammatory cytokines and immunoglobulin in ex vivo cultured pieces from the rheumatoid synovial

membrane.[12] Finally, IL-4 downregulates the surface expression of CD5 on B cells and inhibits spontaneous immunoglobulin and IgM rheumatoid factor production in patients with RA.[13] In summary, IL-4 is a pleiotropic cytokine with potent immunomodulatory functions that affect different cellular targets and are suitable for ameliorating signs and symptoms of chronic arthritis.

## IFN-γ

Production of IFN-γ is the hallmark of Th1 cells. In its biologically active form, IFN-γ is a 34 kDa homodimer that possesses two N-glycosylation sites. IFN-γ is produced mainly by activated T cells and NK cells and has receptors on virtually all cells of the human body. Thus, IFN-γ exerts a multitude of biological functions. The ability of IFN-γ to activate endothelial cells and macrophages is the basis for defining IFN-γ as a pro-inflammatory cytokine. IFN-γ stimulates the production of NO and potentiates the respiratory burst responsiveness of macrophages. It increases the expression of MHC class II molecules and thereby enhances the cells' ability to present foreign antigens. IFN-γ upregulates the expression of the high-affinity Fcγ receptor I on monocytes and neutrophils, which, upon binding to immunoglobulin, stimulates their phagocytic effector functions. On endothelial cells, IFN-γ augments the expression of the adhesion molecule, intercellular adhesion molecule-1 (ICAM-1, CD54), that increases their adhesiveness for leukocytes. IFN-γ production by T cells can be elicited by various stimuli, such as trauma or antigen-specific activation during infections or autoimmune diseases. It has been documented that monocyte-derived IL-12 is probably the most potent inducer of IFN-γ secretion. Recently, IL-18 has been found to be able to induce IFN-γ production from T cells, and numerous studies have now demonstrated the critical role of IL-12 and IL-18 for optimal in vivo induction of IFN-γ by exogenous agents. Several experimental autoimmune diseases are exaggerated by exogenous IFN-γ, although this is not a uniform effect of IFN-γ in different models. Nevertheless, the potent pro-inflammatory activities of IFN-γ combined with its inhibitory potential for the development of Th2 cells make IFN-γ a central mediator of the signs and symptoms of chronic autoimmune inflammation.

## IL-10

IL-10 is a homodimeric cytokine of 17 kDa that was discovered as a potent inhibitor of macrophage effector functions. It is produced by activated monocytes, NK cells, B cells and T cells. In mice, IL-10 is clearly a Th2 cytokine; however, in humans, IL-10 can be produced by both the Th1 and Th2 subsets. IL-10 is able to ameliorate potential pathological autoimmune inflammation through the inhibition at various facets of the immune response. IL-10 inhibits the production of pro-inflammatory cytokines by macrophages, such as IL-1β IL-6, IL-8, IL-12 and TNF-α, and upregulates the production of IL-1Ra and soluble p55 and p75 TNF-receptors. IL-10 downregulates the expression of activating and co-stimulatory molecules on monocytes and dendritic cells, such as MHC class II molecules, ICAM-1, CD80 and CD86. It also reduces the generation of NO, superoxide and prostaglandin $E_2$ in macrophages. In T cells, IL-10 inhibits production of IL-2 and IFN-γ and also blocks T cell proliferation. Thus, IL-10 has potent anti-inflammatory functions and has, consequently, been used in the treatment of experimental autoimmune diseases with great success. For example, IL-10 reduces joint swelling, cellular infiltration, pro-inflammatory cytokine production and cartilage degradation in CIA.[14] Most interestingly, IL-4 and IL-10 synergistically reduce joint

inflammation in acute and chronic arthritis models.[15] Thus, IL-10 might be an effective means for downregulating human chronic autoimmune inflammation by counteracting IFN-γ-mediated pro-inflammatory activities. It should be emphasized, however, that in humans IL-10 cannot be assigned to a particular Th subset. Moreover, and of potential interest for human autoimmune diseases, it has recently been shown that IL-10 is produced by, and induces, the development of regulatory T cells in animals that appear to be most important in maintaining peripheral tolerance.[9]

## EXPERIMENTAL EVIDENCE FOR A DOMINANT TH1 DRIVE IN ANIMAL MODELS OF RA

Murine CIA is an autoimmune diseases in which immunization with cartilage derived type II collagen (CII) induces a T cell response to CII and, additionally, the manifestation of a destructive inflammatory response in affected joints. In many aspects, CIA resembles the immunopathogenesis of RA and has been widely used as a model system for the human disease. However, CIA is more complex in that it appears to have a biphasic course. The first phase at disease initiation is strictly T cell-dependent and is under the control of T cell-derived cytokines, whereas the continuation of the inflammation in the second phase is strongly mediated by humoral immunity. We will present the evidence for a Th1-mediated initiation of the autoimmune response.

When cytokine levels of cultured draining lymph node cells from mice with CIA were monitored during induction and throughout the time of clinical manifestation, IFN-γ was detected in lymph node cell cultures as early as 6 days after immunization, and the addition of CII to the culture medium resulted in an approximately tenfold increase in IFN-γ production. In contrast, Th2 cytokine production was almost completely suppressed 6 days after immunization, indicating that a predominant Th1 response had become manifest at that time. IFN-γ was further increased at the time of clinical manifestation of the disease. IL-4, although present, was found to be markedly suppressed in lymph node cell cultures containing CII. These findings indicate that Th1 responses are prevailing at the time of onset of arthritis and that the activation of CII specific Th1 cells may result in suppression of Th2 activity.[16]

Mice treated with IL-12 or IL-18, two potent inducers of Th1 differentiation in mice, developed significantly higher incidence and more severe disease compared with controls. Cytokine treatments led to markedly enhanced synovial hyperplasia, cellular infiltration, and cartilage erosion. These effects were even more pronounced by combination treatment with IL-12 and IL-18.[17]

Conversely, knockout mice that were deficient in producing biologically active IL-12, manifested a significant reduction in both the incidence and the severity of CIA. Concomitant decreases were observed in serum levels of pathogenic, CII-specific IgG2a antibodies and collagen-induced secretion of IFN-γ by immune splenocytes in vitro, consistent with an impaired Th1 response.[18] Similarly, when neutralizing antibodies to IL-12 were employed in an attempt to block Th1 cell generation, a dramatic attenuation of the severity of the disease, both clinically and histopathologically was observed. This regime was associated with reduced IFN-γ levels produced by ex vivo CII stimulated draining lymph node cells. These findings confirm that IL-12 has a major role in the induction of murine CIA and suggests that this disease is propagated in part by cells of the Th1 phenotype.[19]

## CLINICAL AND EPIDEMIOLOGICAL EVIDENCE FOR A DOMINANT TH1 DRIVE IN RA

For several decades, clinical observations have highlighted an ameliorating effect of pregnancy on the course of RA.[20] In fact, the effect of pregnancy on RA activity is greater than the effect of some of the newer therapeutic agents. Pregnancy improves the symptoms of RA in about 75% of patients, leading to a significant resolution of inflammation and a relief of symptoms which enables the patients to taper substantially or even stop the use of medications. The mechanisms for this phenomenon are still unclear; however, a marked decrease in Th1-mediated immunity during pregnancy has been firmly established. For example, pregnant women have a higher incidence of infections compared to non-pregnant females–in particular, infections with intracellular pathogens. The characteristic Th1 immune reaction, DTH, is diminished during pregnancy. In mice, antigen-specific activation of spleen and popliteal lymph node cells yielded reduced IFN-γ and increased IL-4 and IL-5 responses in pregnant animals compared to controls. Moreover, after in vivo challenge with *Leishmania* antigens, serum levels of IgG1 were elevated in the pregnant mice as opposed to an increase of IgG2a in infected, but non-pregnant controls.[21] Together, the data support the hypothesis that pregnancy induces a shift from Th1 to Th2 immune responses, increasing anti-inflammatory cytokines, which may contribute to the gestational amelioration of RA. Interestingly, relapses of RA occur within 6 months postpartum in 90% of cases. At that time, pregnancy-associated alterations in Th subset activation can no longer be found[20], suggesting that the beneficial Th2 shift has receded and has allowed the Th1-dominated autoimmune inflammation to re-occur.

Several studies have demonstrated a decreased prevalence of allergic diseases in patients with RA. The prevalence of hay fever in patients with RA is significantly lower than it is in appropriate controls (4 versus 8%).[22] Moreover, those patients with RA who have hay fever have less severe disease compared with control patients with RA (without hay fever), as determined by the erythrocyte sedimentation rate, C-reactive protein (CRP) level, joint score, and radiographic joint damage score.[22] As expected, the atopic RA patients had higher levels of serum IgE and peripheral blood eosinophils, but their T cells produced less IFN-γ after maximum in vitro stimulation.[22] In a different study, the incidence and point prevalence of atopy was lower among patients with RA than in control subjects (3.5 versus 16.2%) and the cumulative incidence of atopy was significantly lower for patients with RA (7.5%) than for controls (18.8%).[23] As allergy is the prototype Th2 disease, and activated Th2 cells are able to inhibit both the generation and the function of Th1 effectors (Figure 1), these studies support the notion that the occurrence of a Th2-driven inflammation might be beneficial in RA by inhibiting Th1-mediated immunity.

Exogenous cytokines have been used increasingly in recent years for the treatment of several different malignancies and viral infections. These therapies, some of which are still experimental, provide an excellent opportunity to explore the effect of cytokines on T cell function and differentiation after in vivo application with regard to autoimmunity. IL-12 is a strong inducer of Th1 cell development and subsequent IFN-γ production. Thus, in an attempt to enhance anti-tumour cellular cytotoxicity, IL-12 has recently been used as an experimental treatment for different forms of cancer. When IL-12 was applied to a woman with metastatic cervical cancer, a severe exacerbation of her RA was noted.[24] In contrast to IL-12, IFN-α has already been widely used because of its anti-viral and anti-tumour properties. Like IL-12, IFN-α is a potent inducer of Th1 differentiation in humans. The incidence of autoimmune diseases associated with IFN-α

treatment ranges between 4 and 19%, and several authors have noticed the first onset of a newly established RA or an exacerbation of a pre-existing RA.[25] Together, these observations following the application of Th1-inducing cytokines into patients with malignancies or viral infections strongly emphasize the role of differentiated Th1 effectors in the pathogenesis of RA.

A final argument for the dominant role of Th1 effectors in RA derives from an interesting study that aimed to correlate the Th1/Th2 ratio in the peripheral blood from patients with RA with the clinical course. Although no apparent correlation was detected with disease activity score or CRP at baseline, the initial Th1/Th2 ratio correlated well with the disease activity score 9 months after the beginning of treatment with disease-modifying anti-rheumatic drugs (DMARDs).[26]

## EXPERIMENTAL EVIDENCE FOR A DOMINANT TH1 DRIVE IN RA

Different experimental strategies have been pursued to test the hypothesis of a Th1-dominated inflammation in RA. Although the findings from the majority of reports were in favour of this hypothesis, the results from a number of studies appear not to support a pathogenic role of pro-inflammatory Th1 cells in RA. Several reasons related to the selection of patients and the activity and duration of their disease, the concomitant treatment, a small sample number in combination with the fact that RA comprises different disease entities, and–last but not least–technical difficulties might have contributed to the divergent results. Because of these problems, the interpretation of those studies is difficult. We will concentrate on presenting those data that provided arguments for a contribution of Th1 cells and their cytokines to RA. We are aware of the fact that categorizing a complex disease such as RA just as Th1 or Th2 is probably incorrect and too simplistic. However, an overwhelming body of evidence has been accumulated to suggest that rheumatoid inflammation can be characterized by the presence of activated Th1 effector cells. Moreover, it is generally accepted that the dominance of activated Th1 cells is more prominent at sites of inflammation and more difficult to detect at distant sites, such as the peripheral circulation.

T cells cloned from the human rheumatoid synovial membrane functionally represent the Th1 subset. Out of a panel of 19 synovial membrane derived T cell clones, 18 produced large amounts of IFN-$\gamma$ whereas IL-4 was absent or present in minimal amounts only.[27] In a different study, 15 out of 26 $CD4^+$ $\alpha\beta$ T cell clones from the rheumatoid synovial fluid, the synovial membrane and the peripheral blood from five RA patients produced IFN-$\gamma$ but no IL-4 upon challenge with their specific antigens. Some of those clones produced various amounts of IL-10. Among 11 clones with unknown antigen specificity, seven showed a Th1-like pattern.[28]

Analysis of synovial biopsies by in situ hybridization, immunohistochemistry or RT-PCR revealed that IFN-$\gamma$ could be detected in the majority of specimens, whereas IL-4 was rarely found.[29,30] Interestingly, a significant frequency of those IFN-$\gamma$-producing synovial $CD4^+$ T cells apparently has a distinct cytokine secretion profile as typified by their ability to produce IL-10.[31] The functional importance of this phenomenon warrants further elucidation. Importantly, however, synovial fluid and synovial tissue-derived T cells express activation markers on their surface, indicating their contribution to tissue inflammation. The frequency of IFN-$\gamma$ producing $CD4^+$ T cells is significantly increased in the synovial fluid compared to the peripheral blood[32], resulting in a markedly elevated Th1/Th2 ratio in the synovial fluid that correlated with disease activity.[33] Similarly, a drastically reduced synthesis of IL-4 and IL-10 mRNA in synovial fluid

mononuclear cells of RA patients correlated with disease activity.[34] Finally, when synovial fluid T cells were cultured in the presence of IL-4, they were remarkably stable and resistant to Th2 inducing priming conditions.[32] Together, these data strongly suggest that $CD4^+$ T cells from the inflamed rheumatoid synovium represent activated Th1 cells which secrete IFN-γ and which, in turn, might orchestrate synovial inflammation.

In an attempt to assess the in vivo cytokine profile from $CD4^+$ T cells in RA, and to avoid ex vivo manipulations such as stimulation or prolonged times of culture, a sensitive and reproducible RT-PCR method for analysing cytokine mRNA levels in freshly isolated–not *ex vivo* stimulated–peripheral blood mononuclear cells (PBMC) was developed. Out of 14 RA patients, seven had increased levels for IL-2 compared with healthy controls and five for IFN-γ, implying the presence of activated Th1 cells in the peripheral circulation of patients with RA. Of note, three patients had elevated levels of IL-4.[35] Most interestingly, when re-entry of circulating T cells into sites of inflammation was blocked by a monoclonal antibody (mAb) to ICAM-1, a significant increase in IFN-γ mRNA levels in the PBMC was detected; this might reflect a redistribution of activated Th1 cells from sites of inflammation into the peripheral circulation.[35]

Increased numbers of PBMC secreting IFN-γ and IL-2 were found in patients with new onset synovitis (< 1 year duration). Most interestingly, the frequencies of IFN-γ-secreting PBMC in early arthritis correlated with disease activity, emphasizing the role of Th1 cells in the initiation of the disease.[36]

## EXPERIMENTAL EVIDENCE FOR A BENEFICIAL EFFECT OF INDUCTION OF TH2 IMMUNITY IN ANIMAL MODELS OF RA

Experimental autoimmune diseases provide the opportunity for testing hypotheses of disease development by specifically interfering with particular aspects of the pathogenesis. Because of the strong evidence for a contribution of Th1 cells and their cytokines in the first phase of CIA, an attempt was made to modulate the Th1/Th2 balance in favour of the later. Different approaches, such as Th2 cytokines, inhibitors of Th1 cytokines or in vitro and in vivo engineered cells that were genetically manipulated to produce IL-4 were all applied with convincing success, supporting the hypothesis that interference with an ongoing or established pro-inflammatory Th1 drive by means of activated Th2 cells or their secreted cytokines can prevent or interrupt chronic autoimmunity, synovial inflammation and tissue destruction.

Continuous administration of exogenous IL-4 significantly delayed the onset of arthritis and suppressed clinical symptoms in murine CIA. Several days after cessation of treatment, arthritis occurred; however, joint damage and inflammation at the histological and cellular levels were less severe than those in untreated controls.[10] A similar effect has been observed in another study where IL-4 resulted in suppression of disease activity and strongly reduced cartilage pathology, as determined by histological examination. Moreover, cartilage oligomeric matrix protein (COMP) serum levels were significantly reduced, confirming decreased cartilage involvement. In addition, both histological and radiological analysis showed that bone destruction was prevented, suggesting a potential therapeutic value of IL-4 in inflammatory arthritis.[37] In DBA/1 mice, IL-10 was more effective than IL-4 in suppressing CIA, but, most interestingly, the combination of IL-4 and IL-10 caused pronounced protection against cartilage destruction, even when treatment was started after the initial inflammation had already become manifest.[15]

Recent advances in the understanding of molecular biology have allowed the modulation of target cells for the constitutive production of particular proteins in vivo by means of gene transfer into the target cells. Joint erosion in CIA could be inhibited by intra-articular administration of an adenoviral vector expressing IL-4.[38] Similarly, intradermal inoculation of a plasmid CMV vector encoding IL-10, a Th2 cytokine in mice, 1 week prior to the first immunization with CII reduced foot pad thickness associated with less severe histopathological changes and resulted in lower IgG2a/IgG1 ratios of antibodies to CII than in mice inoculated with the backbone vector or with no vector.[39] Mice that received a subcutaneous inoculation of Chinese hamster ovary fibroblasts transfected with the mouse IL-13 or IL-4 genes experienced a significant reduction in incidence and severity of CIA compared with control groups receiving non-transfected cells.[40] In a more specific attempt to intervene with the induction of a Th1 response by providing a milieu that promotes Th2 differentiation and inhibits Th1 development, dendritic cells were infected in vitro with an adenovirus encoding IL-4. Intravenous injection of infected dendritic cells into mice with established CIA resulted in almost complete suppression of disease, with no recurrence for up to 4 weeks post-treatment. Administration of IL-4-infected dendritic cells decreased the level of specific antibodies to CII, in particular the IgG2a Th1 isotype 14 days after treatment. In culture, spleen cells from the treated mice produced less IFN-$\gamma$ after stimulation by CII than did control groups.[41]

Application of antigens via the nasal or the gastrointestinal mucosa has been shown to induce specific T cell responses with an immunomodulatory phenotype.[42] The regulatory cells that mediate active suppression after nasal or oral antigen administration act via the secretion of suppressive cytokines such as TGF-$\beta$ and IL-4. Intranasal application of denatured CII or trypsin-digested bovine CII before immunization with CII strongly delayed the onset of CIA. The mice administered denatured or digested CII possessed much lower titres of anti-CII IgG2a than did the control mice, whereas titres of anti-CII IgG1 and IgG2b were unchanged or slightly decreased.[43] IFN-$\gamma$ production was significantly reduced upon re-challenge 70 days after a booster immunization. T cell lines established from fed or nasally treated mice showed a pattern of cytokine production involving IL-4, IL-10 and TGF-$\beta$, whereas T cell lines from the control group produced more IFN-$\gamma$ and IL-2. The results demonstrate the induction of antigen-specific Th2 and TGF-$\beta$-secreting regulatory T cells following both oral and nasal treatment, which is associated with suppression of local inflammation in the joints and decreased Th1 responses in the periphery throughout the disease.[44]

Additional support for a beneficial effect of Th2 cytokines in the treatment of CIA derives from treatment studies employing non-depleting mAbs to CD4. Anti-CD4 mAbs not only prevented the induction of arthritis but also significantly reduced serum levels of IgG2a anti-CII Abs. Lymph node cells of treated animals had a reduced production of IFN-$\gamma$ but increased IL-4 upon in vitro challenge with CII. Thus, the results demonstrate that in vivo modulation with non-depleting anti-CD4 mAbs prevents CIA, probably by altering the functional profile of Th1 T cells to Th2.

## EXPERIMENTAL EVIDENCE FOR A BENEFICIAL EFFECT OF THERAPEUTIC INDUCTION OF TH2 IMMUNITY IN RA

In humans, few studies have been performed with the goal of ameliorating RA by modulating the Th1/Th2 balance. Intravenous application of IL-4 was not associated with clinical benefit; however, at the doses required to attain trough levels that were thought

to be sufficient to induce Th2 differentiation in the lymph nodes, IL-4 was associated with significant side-effects. IL-10 was inefficient in RA, which is probably related to the fact that IL-10 in humans is not a Th2 cytokine and does not have prominent Th2-inducing effects. Gene therapy has been performed only in selected RA patients, and no controlled attempt has been made to modify T cell function. Nevertheless, several arguments have been advanced that might suggest that a shift in the balance of Th1/Th2 effector cells is associated with clinical benefit and, moreover, might inhibit chronic Th1 inflammation in RA.

Several studies have reported that DMARDs might be effective in RA at least in part because of their immunomodulatory effects on Th cell subsets. For example, we have recently demonstrated that leflunomide selectively decreased the activation of pro-inflammatory Th1 cells while promoting Th2 cell differentiation from naive precursors.[45] Sulfasalazine potently inhibited the production of IL-12 in a dose-dependent manner in mouse macrophages stimulated with LPS. Importantly, pre-treatment of macrophages with sulfasalazine either in vitro or in vivo reduced their ability to induce IFN-γ and increased the ability to induce IL-4 in antigen-primed $CD4^+$ T cells.[46] Methotrexate significantly decreased the production of IFN-γ and IL-2 in in vitro stimulated PBMC while increasing the concentration of IL-4 and IL-10.[47] Similarly, clinical efficacy of cyclosporin was associated with decreased serum levels of IFN-γ, IL-2 and IL-12 and with significant increases in IL-10. IL-4, however, was not altered.[48] Bucillamine decreased the frequency of IFN-γ-producing $CD4^+$ T cells among generated $CD4^+$ T cells after priming culture of PBMC.[49] Finally, recent reports have suggested that glucocorticoids may inhibit cytokine expression at least in part indirectly through promotion of a Th2 cytokine secretion profile, presumably through their action on monocyte activation.[50] Together, these data suggest that a number of current treatment modalities in RA exert their anti-inflammatory effects by inhibiting Th1 cell activation and/or differentiation and by favouring Th2 differentiation, thereby shifting the Th1/Th2 balance towards the Th2 direction.

As in the mouse, a non-depleting mAb to CD4 effectively reduced signs and symptoms of inflammation and induced clinical benefit in patients with RA.[51] Administration of the anti-CD4 mAb was associated with a substantially decreased potential of peripheral blood T cells to secrete IL-2 and IFN-γ in response to anti-CD3 stimulation in vitro. These data suggest that clinical improvement in patients with RA treated with a non-depleting mAb to CD4 may be related to a decrease in the function of Th1 cells.

Future studies will show whether a shift in the Th1/Th2 balance in humans can be achieved and is sufficient to interrupt established chronic Th1-mediated immunity. It can be speculated that those studies will be performed fairly soon as tools for the safe modulation of Th function in vivo will shortly become available.

## TH1 CELLS IN RA: VILLAINS OR GUILT BY ASSOCIATION?

The arguments depicted herein in detail convincingly demonstrate that Th1 cells and their cytokines are not only present in RA but contribute to the perpetuation of chronic inflammation. However, in contrast to CIA, the data do not yet allow us to conclude whether Th1 cells are the creators of rheumatoid inflammation or rather appear as a consequence of it. In order to delineate the mechanisms underlying the dominant Th1 drive in RA, we have recently shown that isolated memory $CD4^+$ T cells from the majority of patients with early RA manifest a profound inability to

mount Th2 responses.[52] Thus, those patients cannot generate immunoregulatory Th2 cells which might allow ongoing Th1 inflammation to persist and evolve into chronic inflammation, characterized by the continuous activation of T cells, macrophages, fibroblasts and osteoclasts and, subsequently, the destruction of tissue. As this functional abnormality is evident at the time of initial clinical symptoms of arthritis, the data suggest that Th1-dominated immunity is the basis that permits rheumatoid inflammation to develop, but is not the consequence of it. An interesting finding has been made recently that might shed light on the mechanisms contributing to the Th1 drive in RA. T cells from mice transgenic for the human RA associated HLA allele, HLA-DRB1*0401, produced significantly more IFN-$\gamma$ and TNF-$\alpha$ in response to stimulation with the same specific antigen, gp39, than did T cells from mice transgenic for another HLA allele, HLA-DRB1*0402, that is not associated with RA.[53] These data indicate that disease-associated HLA-DR molecules may in fact favour increased Th1-driven immune responses. As T cells recall expression of cytokines they had been instructed to express as a result of previous activation by somatic imprinting of cytokine genes[54], it is possible that antecedent recognition of antigen presented in the context of disease-associated HLA-alleles primed the responding T cells to modify their functional repertoire, predisposing to Th1 cell differentiation after subsequent stimulations. Future studies will have to be carefully conducted to verify the hypothesis that rheumatoid inflammation is indeed caused by activated Th1 cells. However, there is no longer any doubt that Th1 cells and their cytokines substantially promote many aspects of synovial inflammation and that interference with their activation and with the activity of their secreted cytokines might be beneficial in the treatment of RA.

## SUMMARY

Human $CD4^+$ T helper cells can be divided into at least two different subsets. Th1 cells produce the pro-inflammatory cytokines IL-2, IFN-$\gamma$ and LT, activate macrophages and mediate cellular immunity. Th2 effectors down-modulate macrophage activation by secretion of the anti-inflammatory cytokines IL-4 and IL-5, they promote humoral immunity and are involved in allergic inflammation. Imbalances in the ratio of activated Th1 versus Th2 cells have been associated with the development of a variety of pathogenic inflammatory responses, and it has been shown that Th1-mediated immunity is associated with the pathogenesis of several organ-specific autoimmune diseases in animals. For example, CIA is dependent on Th1 differentiation as it can be blocked by IL-4 or IL-10 and intensified by IL-12 or IL-18 but is significantly reduced in IL-12 knockout mice that are compromised in generating Th1 cells. Moreover, modulation of the immune response from Th1 to Th2 ameliorates established disease. In RA, convincing arguments, both clinical and experimental, have been provided to suggest that autoimmune rheumatoid inflammation is also driven by activated Th1 effectors without sufficient Th2 generation to downregulate inflammation. Furthermore, recent data suggest that several treatment modalities currently employed in RA, exert their immunomodulatory effect at least in part by inhibiting Th1 cell activation and/or differentiation and by favouring Th2 differentiation, thereby shifting the Th1/Th2 balance towards the Th2 direction. Thus, selective manipulation of Th cell differentiation to induce Th2 effectors might be a successful approach for interrupting ongoing and established Th1-driven chronic autoimmune diseases such as RA.

**Research agenda**

- definition of molecular mechanisms regulating Th subset differentiation will provide not only new insights into the pathogenesis of rheumatoid arthritis but will also reveal novel targets for immunotherapy
- blocking of Th1 effector activation might inhibit perpetuation of chronic inflammation in vivo
- generation of Th2 effectors might prevent initiation and continuation of autoimmune inflammation in RA
- attempts to modulate the Th1/Th2 balance in chronic inflammation with a therapeutic intention will be performed shortly based on the increasing knowledge of molecular and cellular biology. These studies will have to be carefully designed and monitored to test the hypothesis that a shift in Th1/Th2 activation can be induced and might be beneficial in RA
- several ways to modulate the Th1/Th2 balance have been explored in animal models; the most effective and feasible way in humans will need to be defined

## Acknowledgements

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# Cytokine therapy in rheumatoid arthritis

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## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease and although systemic inflammation manifestation is usually observed, it predominantly affects peripheral joints. T cells and macrophages are the most abundant cells found in the synovial membrane of RA joints [39]. There is indirect evidence that T cells but not B cells may be involved in the pathogenesis of RA since patients with agammaglobulinemia still develop RA [30, 35], whereas leukapheresis which removes T cells from the body was shown to be an effective treatment of RA [79]. Furthermore, patients with RA developing AIDS improve according to the decrease of $CD4^+$ cells. The treatment of RA patients with anti-CD4 antibody therapy is discussed elsewhere (Panayi et al.) in this volume.

Soluble factors such as interferon-γ (IFN-γ) and direct cell/cell contact between activated T cells and macrophages may help to stimulate macrophages to express proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) [38, 72]. TNF-α plays a pivotal role in the proinflammatory "cytokine cascade" regulating the secretion of other proinflammatory cytokines such as interleukin-1 (IL-1) and IL-6 which are both detectable in synovial membranes and synovial fluid in active disease (see chapter by Brennan et al. in this volume). TNF-α also enhances the production of chemokines such as IL-8 and monocyte chemoattractant protein-1 (MCP-1) reviewed in the chapter by Koch and Streiter (this volume) and of adhesion molecules such as E-selectin and ICAM-1 (reviewed in the chapter by Lipsky) to promote chemotaxis and migration of immune cells to the site of inflammation.

TNF-α induces the synthesis and secretion of matrix metalloproteinases (MMPs) including MMP-1 (collagenase-1) and MMP-3 (stromelysin) which may contribute to the degradatory process seen in cartilage [7, 8, 17]. In addition, TNF-α can also induce bone resorption through stimulation of osteoblast/osteoclast activation [77].

TNF-α stimulates the secretion of vascular endothelial growth factor (VEGF) (reviewed in the chapter by Fava and Paleolog) and it was of interest that anti-TNF-α antibody treatment of RA patients led to a significant decrease in VEGF levels in the

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serum of these patients [65]. VEGF is a potent stimulator of angiogenesis and it increases blood vessel permeability and is probably an important mediator in the pannus formation in RA. From these data it is evident that TNF-α is a major player in the complex network of cytokines involved in the inflammatory process in RA and, therefore, is a promising therapeutic target (see the chapter by Feldmann and Maini).

Standard therapy for RA is largely empirical and more than 50% of patients are unable to work within 10 years of disease onset [85]. Moreover, RA is associated with an increased mortality with the current treatment tools available [69]. Throughout recent years the expression of cytokines with anti-inflammatory and immunoregulatory properties have been described in joints of RA patients. These cytokines include IL-10, IL-11, and transforming growth factor-β (TGF-β) [6, 34, 42]. Blocking experiments in RA synovial membranes abrogating the bioactivity of these anti-inflammatory cytokines highlight the important role of these cytokines in limiting the inflammatory process. Thus, it seems to be reasonable to investigate whether cytokines with anti-inflammatory and immunoregulatory activities may be effective in the treatment of RA.

## Interleukin-10

IL-10 has been studied thoroughly in recent years and there is mounting evidence in vitro and in vivo that IL-10 has potent anti-inflammatory activities. IL-10 was described initially as a murine Th2 cell product, which inhibited cytokine synthesis (especially IFN-γ) by Th1 cells [62]. More recent studies however, have shown that human IL-10 is not a typical Th2 cytokine, as it is also produced by $CD4^+$ Th0 and Th1 cells, $CD8^+$ T cells, B cells, keratinocytes, various tumour cell lines and in particular monocytes/macrophages [63, 87] (reviewed [61]). Whilst, IL-10 is thought to be secreted from cells via a classical secretory pathway, recent data indicates that IL-10 may also exist as a membrane form [29].

IL-10 inhibits proliferation and cytokine synthesis of mouse and human T cells, stimulated either by antigen, mitogen, or allogeneic cells in the presence of antigen-presenting cells (APC) [18, 21, 26]. While it has been shown that IL-10 directly inhibits proliferation or cytokine synthesis, independent of another cell population, the majority of publications suggest that the inhibition is indirect, mediated by APC [28, 52]. One important aspect is that human IL-10 down-regulates both constitutive and induced expression of HLA-DR, HLA-DP and HLA-DQ expression in monocytes [19]. B7-1 and 2 are the ligands for the important CD28 co-stimulatory pathway in T cells. IL-10 blocks both B7-1 and B7-2 up-regulation on mouse peritoneal macrophages and down-regulates B7-2 on human dendritic cells [9, 10, 22].

IL-10 inhibits the synthesis of pro-inflammatory cytokines from monocytes which include not only IL-1, IL-6, IL-12, IFN-α, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF, but also chemokines such as IL-8 and MIP-1a [18, 27] at both protein and mRNA levels. In addition to inhibiting TNF-α and IL-1 biosynthesis, IL-10 also induces the production of their natural inhibitors. IL-10 induces IL-1 receptor antagonist (IL-1ra) mRNA expression and can synergise with LPS to induce IL-1ra protein release in monocytes. Work here at the Kennedy Institute demonstrated that IL-10 induces the release of both soluble TNF receptors; p55 and p75 in monocytes and in RA mononuclear membrane culture [41]. These results collectively indicate that IL-10 exerts strong anti-inflammatory activities and thus has been termed a 'macrophage-deactivating factor'[4]. This concept is illustrated in Fig. 1.

## Interleukin 10 'deactivates' macrophage function

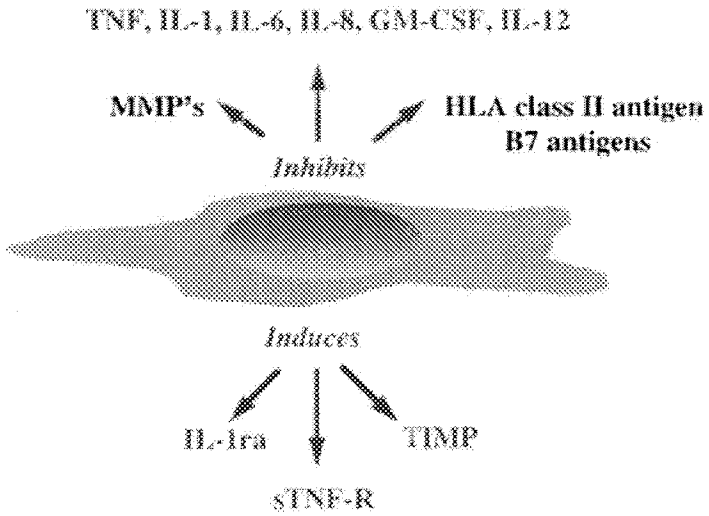


**Fig. 1.** Interleukin-10 (IL-10) is a macrophage-deactivating factor. IL-10 is a potent immunoregulatory molecule deactivating macrophage function principally by inhibiting cytokine synthesis and increasing the production of cytokine inhibitors (TNF, tumour necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MMP, matrix metalloprotease; IL-1ra, IL-1 receptor antagonist; sTNF-R, soluble TNF receptor; TIMP, tissue inhibitor metalloproteinase)

The presence of IL-10 has been documented in RA peripheral blood [50] and synovial joints by reverse transcription-polymerase chain reaction (RT-PCR) of fresh frozen biopsy specimens, immunostaining of fresh frozen biopsy specimens, and by assay of 24-h culture supernatants of dissociated joint cell cultures [16, 42]. Furthermore, we found that in these RA synovial cell cultures the endogenous IL-10 produced is functional, since inhibition of its activity using a neutralising monoclonal antibody enhanced TNF-α and IL-1 production (Fig. 2 a) [42]. Conversely addition of recombinant (r)IL-10 to these cultures inhibited TNF-α and IL-1 production by approximately 50% (Fig. 2 b). In a similar study but using synovial tissue organ cultures [13], it was observed that exogenous IL-10 also inhibited IL-1β, although IL-4 was more potent, and additionally that IL-4 (but not IL-10) induced the production of the native inhibitor of IL-1, IL-1ra. In addition, IL-10 seems to ameliorate the degradatory process of cartilage and bone by inhibiting the synthesis of MMPs such as collagenase in stimulated human monocytes and by increasing the synthesis of the natural MMP inhibitor TIMP-1 (tissue inhibitor metalloproteinase) [46].

These promising in vitro results led to therapeutic animal studies in DBA/1 mice with established collagen-induced arthritis (CIA). Studies in our group demonstrated that recombinant murine IL-10 was therapeutically active in vivo when delivered by a single daily intraperitoneal injection and significantly inhibited clinical disease progression at a dose of 5 μg/day (Fig. 3). Histological analysis confirmed the observed clinical benefit [82]. Additional studies have also demonstrated the therapeutic potential of IL-10 in vivo. A pre-clinical 48-day course of IL-10 treatment at a dose of 100 ng/day was found to significantly suppress the clinical severity of CIA in DBA/1 mice [75]. Importantly, no augmentation of the anti-collagen antibody response was ob-

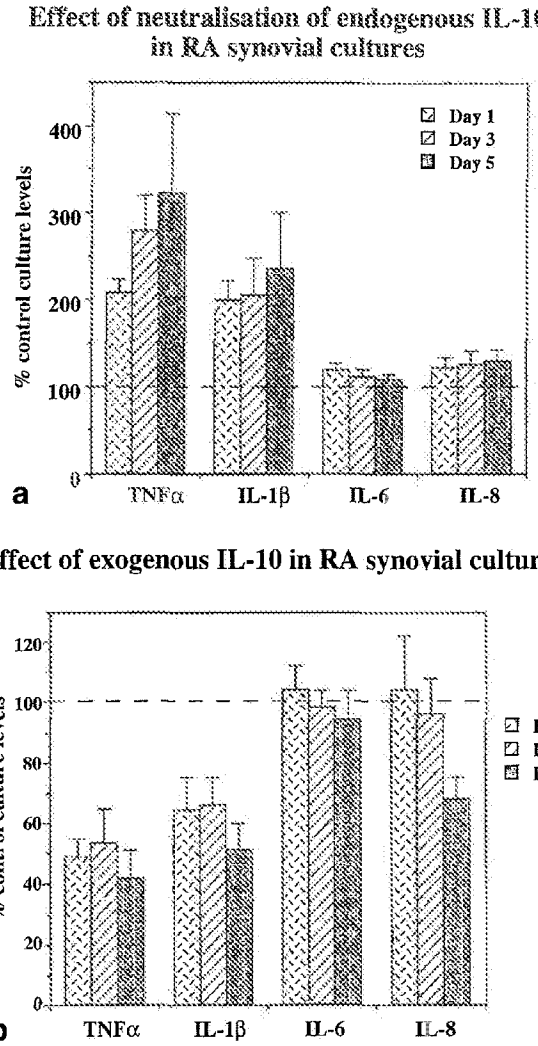


**Fig. 2 a, b.** Neutralization of spontaneously produced IL-10 and addition of exogenous IL-10 in rheumatoid arthritis (RA) synovial membrane cultures. Pooled data from five to ten RA synovial membrane cultures. **a** Neutralisation of endogenous IL-10 with the 9D7 neutralising rat monoclonal anti-IL-10 antibody (2 μg/ml) results in increased levels of TNF-α and IL-1β, but not IL-6 and IL-8. **b** Exogenous rIL-10 (10 ng/ml) decreases TNF-α and IL-1β levels in RA cultures. *Dashed lines* show 100% of control untreated cultures. Reproduced with permission from [42]

served in treated mice. In another study, treatment of established CIA with rIL-10 mildly suppressed clinical disease, however, the clinical benefit was markedly augmented by combination treatment with IL-4 [40]. Accelerated onset of CIA and increased disease severity was observed following treatment with neutralising anti-IL-10 antibodies, suggesting a dominant role for IL-10 in the natural suppression of arthritis in this model system.

Since IL-10 is effective in animal models of arthritis, clearly there has been interest to establish whether IL-10 is also effective in human RA. The safety issues regarding IL-10 have been examined, and IL-10 administered to healthy volunteers in a single intravenous bolus injection demonstrated no clinical adverse event [12]. In vitro studies on isolated peripheral blood mononuclear cells from those IL-10-treated volunteers showed a transient reduction in phytohaemagglutinin-stimulated T cell prolif-

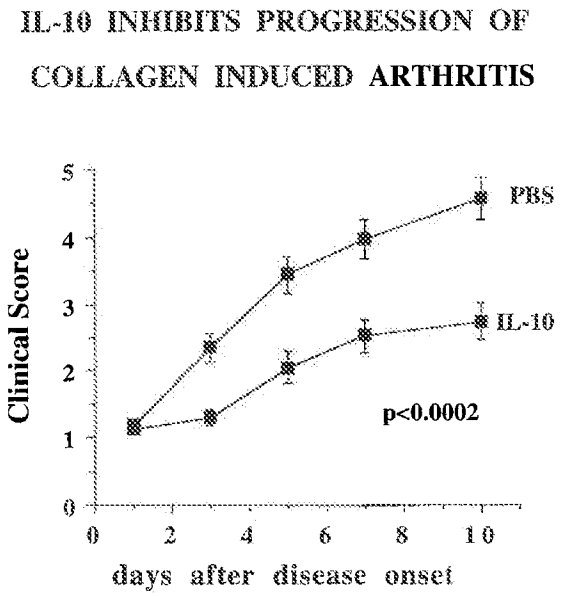


**Fig. 3.** IL-10 inhibits progression of collagen-induced arthritis. DBA/1 arthritic mice were treated with 5 μg/ml recombinant mouse IL-10 from the onset of disease. Data from three separate experiments were pooled. Values are the mean ± SEM. Modified and reproduced with permission from [82]

eration and a marked inhibition of endotoxin-induced IL-1β and TNF-α production, whereas their respective antagonists remained unaffected.

In light of this background, a recent trial of human rIL-10 (rh IL-10) in subjects with active RA was performed [53]. The primary objective of this trial was to assess the safety and tolerability of rIL-10 and, secondarily, to evaluate its effect on disease activity and on cytokine physiology. In this multicenter, randomised, double-blind, placebo-controlled, multiple-dose study, after a 4-week washout period of DMARDs, 72 subjects with active RA received rIL-10 at doses of 0.5, 1.0, 5, 10, 20 mcg/kg or placebo by daily subcutaneous injections for 4 weeks. rIL-10 was clinically well tolerated and no anti-IL-10 antibodies were detected in any subjects at any time during the study. During treatment there was a reduction in mean platelet count in subjects treated with the highest rIL-10 doses, although overall rIL-10 was well tolerated and safe. Despite the beneficial effects of IL-10 in murine CIA, the clinical effects of IL-10 in this trial of human RA were marginal with a beneficial trend towards improvement in RA disease activity at 4 weeks (>20% ACR criteria) only observed in the 5 mcg/kg rIL-10 group. However, subjects receiving doses of rIL-10 required less use of DMARDs during the 8 weeks following completion of therapy. Circulating levels of soluble TNF-R (p55 and p75) as well as IL-1ra showed a significant increase ($P < 0.001$ for the former and $< 0.01$ for the latter) at the highest rIL-10 doses, indicating that the IL-10 was having a biological role in vivo. Future trials are planned exploring different doses within the range observed to have some clinical effect.

While IL-10 may be a good candidate for treatment of RA in humans, we should be aware about the potential drawbacks which can be deduced from other in vitro results. IL-10 exerts potent inhibitory effects on T lymphocytes and on monocyte/macrophages which could lead to higher susceptibility to viral, fungal and bacterial infections in IL-10-treated patients. Patients suffering from RA already exhibit an increased susceptibility to infections compared with controls [60] and additional IL-10 treatment

may further increase infection rates in RA patients. IL-10 treatment may further stimulate immunoglobulin and thus rheumatoid factor (RF) production as treated DA rats with active CIA showed higher autoantibody production to collagen type II than controls [67]. Furthermore, circulating B cells require IL-10 to reach terminal maturation to produce IgM-RF. It is interesting, therefore, that in RA serum, IL-10 levels correlate with IgM-RF titres [16, 66], indicating that IL-10 may stimulate humoral immune responses worsening disease activity correlated with high RF production.

## Interleukin-11

There is increasing evidence that IL-11 is another cytokine with anti-inflammatory activity [70, 78]. We and others were able to detect elevated levels of IL-11 in synovial membrane mononuclear cell culture supernatants and synovial fluid of RA patients [34, 64]. In our studies on RA synovial membrane cell cultures exogenous rhIL-11 decreased TNF-α levels only in the presence of an agonistic soluble IL-11 receptor (sIL-11R). In contrast, blocking the biological activity of endogenously produced IL-11 increased TNF-α production about twofold compared to untreated controls, indicating that IL-11 is active in RA synovial membranes and suppresses TNF-α production. When endogenously produced IL-10 and IL-11 were both blocked in the culture system, TNF-α production increased more than 20-fold (Fig. 4), indicating that both cytokines are acting together in synergy as strong inhibitors of the proinflammatory cascade in vivo [34]. IL-11 not only seems to modulate parts of the proinflammatory cytokine cascade but also to have protective effects on mesenchymal tissue in RA. Exogenous IL-11 dose dependently inhibited the production of MMPs such as MMP-1 (collagenase) and MMP-3 (stromelysin) in RA synovial membrane mononuclear cell cultures and in RA synovial membrane fibroblasts, and reversed TNF-α induced sup-

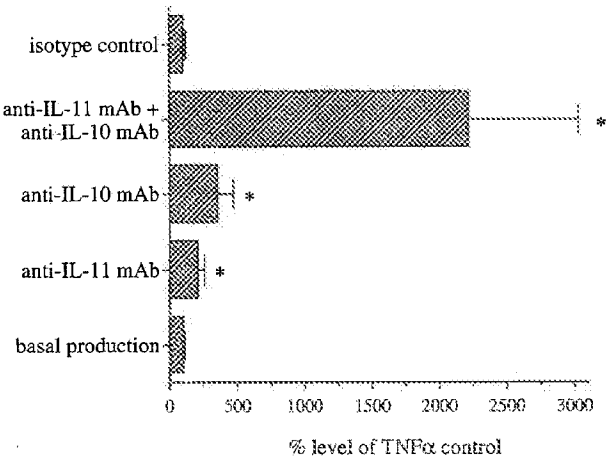


Fig. 4. Blockade of IL-11 activity enhances TNF-α production: synergy with IL-10. RA synovial membrane cells (pooled data from five experiments) were cultured in triplicate, treated for 48 h with neutralising anti-IL-11 mAb (10 μg/ml), neutralising anti-IL-10 mAb (10 μg/ml) or both antibodies together and supernatants tested for TNF-α by ELISA and illustrated as % level of TNF-α control. TNF-α production was significantly increased in cell cultures treated with anti-IL-11 mAb (2-fold), anti-IL-10 mAb (3-fold) or both antibodies (22-fold) compared with basal production, anti-IL-11 mAb- and anti-IL-10 mAb-treated synovial membrane cell cultures, respectively. Modified and reproduced with permission from [34]

pression of TIMP-1 production in RA synovial membrane fibroblasts. These encouraging in vitro results were a rationale for us to investigate the effects of rhIL-11 in the CIA model in DBA/1 mice. rhIL-11 was active in these mice, treated after onset of disease, and doses as low as a single injection of 3 μg/day were shown to significantly attenuate the clinical severity of established disease as well as provide some protection from joint damage as assessed by joint histology [83]; although there was a suggestion at high doses of IL-11 (50–100 μg/day) that the anti-collagen response may have been augmented by treatment, there was no significant elevation of anti-collagen type II antibodies detected at any of the doses used. The therapeutic effect in mice injected with high doses (50–100 μg/day) of rhIL-11 was diminished by the 10th day of treatment but was still visible in mice treated with 310 μg/kg per day of rhIL-11 or less. Immunogenicity of human IL-11 in mice may have contributed to the loss of efficacy in mice treated with higher doses of rhIL-11 because an anti-rhIL-11 response was detected in the latter group. These in vitro and in vivo data suggest that IL-11 has anti-inflammatory properties and may be a candidate for biological treatment of RA. However, provision of IL-11R may be a limiting factor for the efficacy of IL-11 in vivo and the role of sIL-11R in the inflammatory process in RA should be investigated. As IL-11 stimulates megakaryopoiesis and thrombopoiesis it has been used successfully as a therapeutic agent in breast cancer patients with thrombocytopenia after chemotherapy [31, 86]. Adverse events were reversible and oedema, headache, tachycardia and palpitations were the only symptoms reported significantly more often in the rhIL-11-treated group. However, in patients with normal or elevated numbers of platelets in the blood, such as in RA, IL-11 may increase peripheral blood platelet numbers even further and hence enhance the risk for venous and arterial thrombosis. IL-11 also stimulates the production of acute-phase proteins such as fibrinogen in human hepatocytes which may also contribute to a greater risk of thrombotic events in IL-11-treated patients [2]. Clinical trials of IL-11 in RA are ongoing, the results should be informative.

## Transforming growth factor-β

TGF-β, of which there are three isoforms (TGF-β1, TGF-β2 and TGF-β3) is another cytokine with potent immunoregulatory properties. It is released from a variety of cells in an inactive form and binds to cell surface-specific receptors (reviewed [48, 54, 55]). A number of studies from different groups have documented that TGF-β is abundant in both the precursor, inactive, form and the active form in rheumatoid joints [6, 24, 47, 51]. However, while TGF-β is present in abundant amounts in RA synovial fluids and is produced spontaneously by RA synovial membranes, its role in RA still remains elusive. For example, TGF-β inhibits immune functions including T and B cell proliferation [73] and reduces TNF-α, IL-1 and IFN-γ production in peripheral blood monocytes but not in RA synovial membrane mononuclear cells [6, 11]. TGF-β is chemotactic for neutrophils and monocytes and promotes angiogenesis supposedly via up-regulation of vascular endothelial growth factor (VEGF) in vivo [25, 68, 71, 80] and, thus, may contribute to an inflammatory response.

On the other hand, TGF-β is mainly involved in tissue protection and repair by down-regulating the production of MMPs such as collagenase, stimulating the production of TIMP and inducing the synthesis of collagen type I and XI [43, 47, 71, 84]. Thus locally, TGF-β may promote reparative processes in arthritic synovial connective tissue scarring and tissue repair by inhibiting cartilage and bone destruction.

The in vivo effects of TGF-β are also contradictory. Thus, if injected locally into the joints of normal rats, TGF-β resulted in a rapid leukocyte infiltration with synovial hyperplasia leading to synovitis [1], whereas if injected systemically into rodents 'susceptible' to arthritis it antagonised the development of polyarthritis [5, 44]. Furthermore, in a recent publication it was shown that anti-TGF-β antibody, if injected locally into the joint of rats with arthritis diminished the ongoing disease [81]. These studies indicate the multipotential properties of TGF-β, and the differential effects if injected systemically or locally into the joint. The inhibitory and stimulatory effects of TGF-β on cells of the immune system may partly be explained by the different effects of TGF-β on resting and activated cells. Resting cells may be stimulated and activated cells may be inhibited by TGF-β, making TGF-β a regulatory cytokine converting an inflammatory site into one dominated by resolution and repair [56]. Systemic application of TGF-β is an option in chronic inflammatory diseases such as RA, but its activity on inactive immune cells, not involved in the inflammatory process but important for the defence against micro-organisms, may lead to a higher incidence of infections in these patients. Its activity in stimulating collagen production and to inhibit degradation of mesenchymal tissue may lead to fibrotic conditions in organs such as lungs, kidneys and liver with a potentially detrimental outcome.

## Interleukin-4

In common with TGF-β, IL-4 also displays some immunoregulatory effects such as inhibition of lipopolysaccharide-induced IL-1, TNF-α, $PGE_2$ and 92-kDa gelatinase production [23, 32, 45, 76]. However, in contrast to TGF-β, IL-4 has not been found reproducibly in rheumatoid synovial tissue cultures [58], nor in T cells cloned from RA synovial biopsy samples [14], although it has been detected in reactive arthritis [74]. This and other evidence suggests that $CD4^+$ Th2-derived cytokines are not abundant in RA joints, and that $CD4^+$ Th1 cells predominate in this site [57]. It is possible that the lack of IL-4-producing $CD4^+$ Th2 cells contributes to the pathogenesis of RA, and this has led to suggestions that IL-4 may be a useful therapeutic agent ([59] and Mitchison, unpublished observation). The former group demonstrated [59] that the addition of recombinant IL-4 to RA synovial tissue organ cultures resulted in the inhibition of proinflammatory cytokine production. Using dissociated synovial cell cultures [15], we did not observe that the addition of IL-4 had any significant effects on TNF-α levels in RA synovial membrane cell cultures but it enhanced the expression of membrane bound p55 and p75 TNF receptor in RA synovial joint cells, indicating that IL-4 may even enhance TNF-α activity in RA joints. These effects aside, it is interesting to note that IL-4 production in RA joints is defective, and there is a report suggesting that the incidence of allergies is lower in RA patients [49].

IL-4 has also been used for the therapy of CIA, and, as with the human synovial studies, the results are not in full agreement, which may in part reflect differences in cytokine delivery, dosage and therapeutic regimes. Thus, Joosten et al. [40] found that administration of murine recombinant IL-4 (rmIL-4) alone at the day of onset of arthritis *did not* ameliorate disease, whereas the combination of rmIL-4 *and* rmIL-10 showed a more pronounced effect than rmIL-10 alone. In addition, blocking endogenously produced mIL-4 with a neutralising mAb did not change disease activity, whereas neutralisation of IL-4 and IL-10 together increased disease severity. In contrast, treatment of CIA in DBA/1 mice at the time of immunisation with neutralising

anti-IL-4 antibodies exacerbated the arthritis, and DBA/1 mice transplanted with IL-4 gene transfected chinese hamster ovary fibroblasts showed a significantly lower incidence and severity of CIA [36]. It is interesting to note that the transfected IL-13 gene demonstrated similar beneficial effects [3]. IL-13, like IL-4 is produced by activated T cells and, while it shares many properties in common with IL-4 including inhibition of monokine production [20, 33], it does not have any effect on T cells which lack the IL-13 receptor. This property may make IL-13 a more attractive candidate with respect to immunoregulation.

We have recently examined the effect of continuous administration of exogenous IL-4 using an osmotic pump on the development of CIA in DBA/1 mice [37]. Exposure to IL-4 for 28 days significantly delayed the onset of arthritis from 19 to 37 days and suppressed clinical symptoms. Arthritis occurred approximately 13–24 days after treatment ceased. Thereafter, the severity and duration of clinical symptoms were similar to those of control animals, although both joint damage and inflammation at the histological and cellular levels were less severe than untreated controls. During IL-4 treatment, anti-collagen antibody levels were reduced, most significantly those of the IgG2a subclass (Th1 type), histology scores were lower and the most striking effect was a thousandfold decrease in TNF-α secretion by synovial cells. No significant differences in IgE levels (Th2 type) were found between controls and IL-4-treated mice, suggesting that the anti-inflammatory properties of IL-4 are mediated in part by down-regulation of Th1 responses rather than up-regulation of Th2 responses.

In conclusion, while IL-4 may have profound effects on proinflammatory cytokine release in vitro, and some data suggest that it has a therapeutic benefit in arthritic animals, in vivo studies required continuous release of IL-4, either by gene therapy or the protein administered with an osmotic pump. Whether IL-4 will be beneficial in human RA remains to be demonstrated, although trials are in progress.

## Conclusion

The cytokines described above have many anti-inflammatory effects in different in vitro models but the in vivo results are much less encouraging at the moment. Cytokines are regulatory mediators with quite different effects on different cells involved in the inflammatory process, and the so called anti-inflammatory cytokines IL-10, IL-11, TGF-β and IL-4 also have some proinflammatory activities, which may explain the rather poor effects overall seen in some in vivo studies. From the very limited data available, IL-10 seems to be the most promising cytokine to be used as a biological agent in RA. The role of IL-11 in the inflammatory process is only partly elucidated at the moment but from the results presented so far it is also a good candidate as an anti-inflammatory agent in this disease. Our findings, that neutralisation of both IL-10 and IL-11 together dramatically increases the inflammatory potential in RA synovial membrane cell cultures, may indicate that combination therapies should be considered to increase the efficacy of treatments with biological agents. The co-operative/complementary immunoregulatory effects of IL-10 and IL-11 on inflammatory tissue are summarised in Fig. 5.

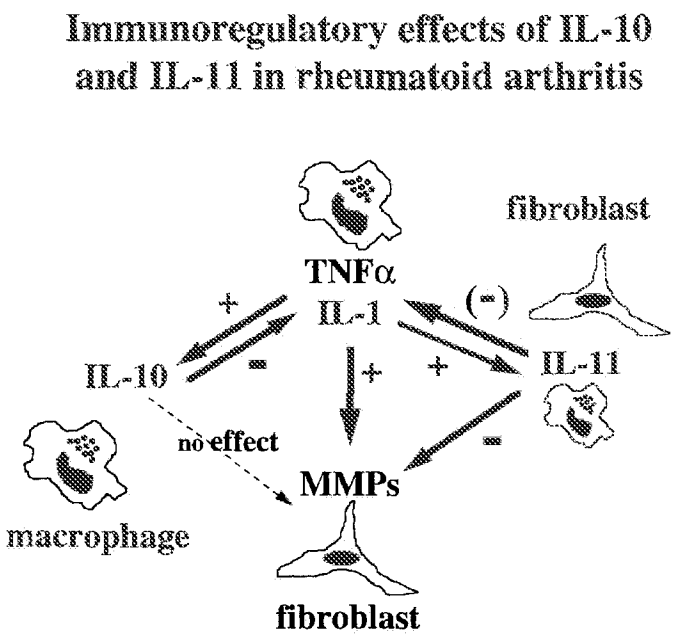


**Fig. 5.** IL-10 and IL-11 interact to regulate pro-inflammatory effects. IL-10 and IL-11 exhibit both overlapping and independent effects of macrophages and fibroblasts in RA synovial tissue to 'down-regulate' cytokines and enzymes with pro-inflammatory effects, and to increase the production of inhibitors

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# Histamine in Rheumatoid Arthritis

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## Abstract

Rheumatoid arthritis (RA) is an autoimmune disease characterized by a persistent inflammation of the synovium, leading to the erosion of articular cartilage and bone. Synovial mast cells and their effector molecule, histamine, receive increased attention as mediators of joint inflammation. The aim of our study was to analyse levels of free histamine in serum and joint fluid of RA patients and to evaluate the potential inflammatogenic properties of histamine *in vivo* and *in vitro*. Histamine levels were measured by an ELISA in synovial fluid and sera of RA patients and of healthy controls. Histamine levels were also assessed in plasma of RA patients undergoing anti-TNF-α treatment. In the murine part of the study, histamine was injected intra-articularly in the knee joint of mice and the joints were subsequently analysed with respect to induction of inflammation. RA patients displayed significantly lower levels of histamine in circulation (0.93 ± 0.16 ng/ml) compared with the healthy controls (1.89 ± 0.45 ng/ml, $P < 0.001$). Locally, in synovial fluid the levels of histamine were even lower (0.37 ± 0.16 ng/ml, $P < 0.0006$). Long-term anti-TNF-α treatment significantly increased circulating levels of histamine in RA patients. Our experiments on animals show that histamine on its own neither induces inflammation in the joint cavity nor influences the course of HMGB1 and peptidoglycan-induced joint inflammation. Based on our experimental and clinical studies we suggest that histamine lacks harmful properties in RA.

## Introduction

Rheumatoid arthritis (RA) is a disease characterized by a chronic inflammation of the synovium, leading to the erosion of articular cartilage and bone [1–3]. The pathophysiological process of RA is often assigned to the actions of fibroblast-like synoviocytes, macrophages, T lymphocytes and their soluble products, such as pro-inflammatory cytokines and metalloproteinases [4–7].

The role of mast cells (MC) in the pathogenesis of RA is largely unknown; yet circumstantial evidence indicates that MC may play a role in the disease process. An increase in the number of MC was noted in the synovial tissue and fluid of patients with RA [3]. It has also been established that a considerable number of synovial specimens displayed MC activation and degranulation at the site of cartilage erosions [6]. Presence of degranulated MC in joint tissue, before any obvious clinical or histological inflammation, suggests a role for MC in the effector phase of inflammatory arthritis [9]. However, the true role of this cell population in the pathogenesis of RA remains unclear.

Histamine is produced by a wide range of cells that are capable to home from the blood to the synovium. However, circulating blood histamine originates almost entirely from basophiles and from MC [3, 10, 11]. Recently, attention has been turned to histamine and its potentially harmful role in RA. There is evidence that histamine is present in both the diseased synovium and the joint fluid [3, 8, 12–14]. It is, furthermore, believed to be synthesized by an array of leukocytes including T lymphocytes, macrophages and neutrophils, and has been proposed to regulate their cytokine production [4, 5, 15]. Histamine has also been detected in articular cartilage of RA patients, which suggests chondrocytes as another synthesis locus. In addition, the expression of histamine and histidine-decarboxylase (HDC) have been observed in many different tissues of the human body, which suggests that histamine has important functions in the regulation of basic biological cell processes [4, 5, 11].

Histamine acts through four different types of receptors (H1, H2, H3 and H4) on various cell types. In the human joint, H1 and H2 receptors are present on the synovial fibroblasts, as well as on articular chondrocytes,

and they exert their effect through two different pathways [16, 17]. This might be one of the reasons why earlier studies concerning histamine, its presence and role in joints have been contradictory. These findings led to the suggestion that histamine increases inflammation in RA and that presence of histamine in synovial fluid (SF) could be a sign of the disease [13, 18, 19]. On the other hand, some studies have emphasized the anti-inflammatory role of histamine through its capacity to switch a Th1 response to a Th2 response, via binding to H2 receptors, and hence upregulating the secretion of anti-inflammatory cytokines, such as IL-4 and IL-10 [20–23]. This is further supported by a study by Garaczi *et al.* [23], where HDC knockout mice (HDC−/−) displayed significantly higher increase of ear swelling when compared with wild-type mice, suggesting that histamine might have a suppressive effect on the production of Th1 cytokines and, consequently, on the inflammatory response. Finally, a growing number of studies suggest that histamine is involved in the downregulation of human polymorphonuclear leukocyte functions in inflammatory responses, such as production of oxygen radicals, cytokine and leukotriene production as well as chemotaxis [24, 26].

*Ex vivo* experiments have shown that lymphocytes from RA patients spontaneously release considerably more histamine than do those from healthy blood donors. Thus, the local release of histamine by lymphocytes and MC at the level of the inflamed joint could be a potential source of this molecule [13]. Several studies concerning levels of histamine in plasma, serum and SF in patients with diverse arthritides have been carried out in the past, with conflicting conclusions regarding the impact of histamine on disease severity [3, 18, 19]. The aims of the present study included: (a) simultaneous measurements of histamine levels in serum/joint fluids in well characterized patients with RA; (b) to study prospectively the impact of efficient anti-rheumatic treatment in circulating histamine levels; and (c) to asses *in vivo* and *ex vivo* the potential pro-inflammatory/arthitogenic role of histamine.

## Materials and methods

*Patients and controls.* Sera and synovial fluid samples were collected from 44 patients with RA (16 males, 28 females, age range 25–84 years) who attended the Rheumatology clinics, at Sahlgrenska University Hospital in Gothenburg, Sweden, for acute joint effusion. RA was diagnosed according to the American College of Rheumatology criteria. The study was approved by the Ethics Committee of the University of Göteborg (S-441-01). Clinical characteristics of RA patients are presented in Table 1. At the time of synovial fluid and blood sampling most of the patients received non-steroidal anti-inflammatory drugs. Recent radiographs of the hands and feet were obtained for all the patients. Presence of bone erosions, defined as the loss of cortical definition at the joint, was recorded in proximal interphalangeal, metacarpophalangeal, carpal, interphalangeal and metatarsophalangeal joints of forefeet. Presence of single erosion was sufficient to fulfil the requirement of an erosive disease. Presence of the rheumatoid factor (RF) of any of immunoglobulin isotypes was considered as positive. Paired blood samples were also collected from 10 RA patients (eight females and two males, age range 24–72 years, disease duration: 0–23 years, methotrexate dose 7.5–22.5 mg/week) before and after five infusions of antibodies against tumour necrosis factor-alpha (anti-TNF-α, infliximab, total dose 1000 mg). Disease activity score (DAS) and C-reactive protein (CRP) in serum were assessed at each sampling occasion.

Table 1 Clinical characteristics of patients with rheumatoid arthritis.

| | Erosive RA, $n = 34$ | Non-erosive RA, $n = 10$ |
|---|---|---|
| Gender (F/M) | 21/13 | 7/3 |
| Age (years) | 61.1 ± 2.3 | 63.7 ± 5.4 |
| Rheumatoid factor (+/−) | 32/2 | 4/6 |
| Disease duration | 14.2 ± 1.5 | 7.3 ± 2.5 |
| Treated with DMARD (%) | | |
| Methotrexate | 20 (59) | 2 (20) |
| Other | 6 (18) | 1 (10) |
| Non-treated | 8 (24) | 7 (70) |

Plasma samples from 33 healthy individuals (two males and 35 females, age range 54–67) were used in the control group. Synovial fluid from 19 patients (11 males and eight females, age range 21–43) with traumatic knee injuries was used as a second control group.

*Measurement of histamine levels.* A sandwich ELISA (Immunotech, Marseille, France) was used to assess histamine levels in plasma and SF samples, according to manufacturer's recommendations. In brief, principle of this ELISA is based on a competition-type assay. Histamine of the samples, standards and controls is chemically modified during an acetylation step using *N*-hydroxysuccinimide ester coupled to succinyl-glycinamid (SGA), subsequently forming a histamine–SGA complex. After incubation of the samples, the wells are washed and the bound enzymatic activity is detected by the addition of a chromogenic substrate. The intensity of the colour development is inversely proportional to the histamine concentration in the sample. The absorbance is read at 405 nm. The histamine concentration in samples was calculated by interpolation from a standard curve that is performed in the same assay as the samples. A computer program (DeltaSoft$^{TM}$) was used to construct a four-parameter curve fit and histamine concentration in the samples was calculated based on that curve.

*Intra-articular injection of histamine, HMGB1 and peptidoglycan.* To assess pro-inflammatory properties of histamine, 24 healthy female NMRI mice were injected intra-articularly (i.a.) in the knee joint with a total volume of 20 μl containing 10, 1, 0.1 or 0.01 ng of histamine (Sigma, St Louis, MO, USA) in physiological saline. Control mice were also injected i.a. with the vehicle solution alone. After 6 and 72 h, three mice from each group were sacrificed and the knee joints were obtained for histological analyses. To evaluate potential anti-inflammatory effect of histamine, healthy NMRI mice were injected i.a. with a mixture of histamine (10 ng/knee) and (a) HMGB1 (2 μg per knee, $n = 16$); or (b) staphylococcal peptidoglycan (10 and 50 μg per knee, $n = 16$). Control mice were injected i.a. with HMGB1 ($n = 16$) or peptidoglycan ($n = 16$) alone. The mice were sacrificed after 72 h and the knee joints were obtained for histological analyses.

The animal study was approved by the Council for Animal Experiments of the University of Göteborg (271-2003).

*Histological examination of knee joints.* A histological examination of the knee joints was performed after fixation, decalcification and paraffin embedding of specimens. Tissue sections were prepared and stained with haematoxylin and eosin. Slides were examined and scored with respect to severity of synovitis as follows: 0 = no synovitis, 1 = mild synovitis, 2 = moderate synovitis and 3 = severe synovitis. All evaluations were performed on coded slides in a blinded manner.

*Preparation and stimulation of spleen cultures.* Mononuclear cell cultures were aseptically obtained from mouse spleens and resuspended in Iscove's complete medium (10% fetal calf serum, $5 \times 10^{-5}$ M 2-mercaptoethanol, 4 mM L-glutamine, 50 μg gentamycin/ml) at final concentration $1 \times 10^6$ cells per ml. The splenocytes were exposed to histamine at concentrations 0–100 μM. To evaluate anti-inflammatory properties of histamine, cell cultures were subjected to histamine treatment (10 μg/ml) overnight and further stimulated with 3 μg/ml of toxic shock syndrome toxin 1 (TSST-1), or 1.5 μg/ml of concanavalin A (Con A) (ICN Biomedicals Inc., Aurora, OH, USA). The supernatants were collected following 24 h stimulation and stored at −20 °C.

*Analysis of interleukin-6 and interleukin-10.* Interleukin-6 (IL-6) levels in the supernatants were measured by a bioassay based on murine hybridoma cell line B13.29, subclone B9, as described previously. This cell line is dependent on exogenously supplied IL-6 for its growth. Interleukin-10 measurement was performed in plasma from anti-TNF-α-treated RA patients. An analysis was carried out with a sandwich ELISA (BioSite, Täby, Sweden), as recommended by the manufacturer.

*Induction of T cell/macrophage and of granulocyte-mediated inflammatory responses in vivo.* Granulocyte-mediated and T-lymphocyte-independent inflammation was induced in mice by injection of 30 μl of olive oil subcutaneously (s.c.) in hind left paw [27]. Paw swelling was registered with an Oditest spring caliper (Kroplin, Hessen, Germany) before and 24 h after the injection. The inflammatory response was expressed as the increase in paw thickness in $10^{-3}$ cm. To study T-cell/macrophage-dependent inflammation, mice were sensitized by epicutanous application of 150 μl of a mixture of 95% ethanol and acetone (3:1) containing 3% 4-ethoxy-methylene-2-phenyloxazolone (OXA) (Sigma) on the abdomen skin [28]. Seven days after the sensitization all mice were challenged by topical application of 15 μl of 1% OXA dissolved in acetone and olive oil on both sides of the right ear. The thickness of the ear was measured before and 24 h after challenge using an Oditest spring caliper. The intensity of delayed-type hypersensitivity (DTH) reactions was expressed as (ear thickness 24 h after challenge − thickness before challenge) × $10^{-3}$ cm units. Two weeks after the initiation of OXA-induced inflammation, mice were bled and serum was stored at −20 °C. Levels of anti-oxazolone antibodies were measured using an ELISA.

*Pre-treatment with sodium cromoglycate.* One day prior to sensitizing the mice with OXA or injection with olive oil, eight male NMRI mice were treated intraperitoneally with 200 μl of sodium cromoglycate (SC; 7.7 mg/ml in PBS) (Lomudal®, Aventis Pharma AB, Stockholm, Sweden), which is an MC membrane-stabilizing agent. This treatment regime has been previously shown to efficiently reduce the levels of histamine in mice [29]. Another 10 mice served as controls and received the vehicle solution.

*Analysis of oxazolone antibodies in sera.* Ninety-six-well microplates (NUNC, Roskilde, Denmark) were coated over night at 4 °C with OXA conjugated to dog albumin (DSA-OXA) (0.003%) dissolved in PBS.

After washing three times in PBS and blocking with 0.5% BSA (Sigma) dissolved in PBS, individual sera were serially diluted in BSA–PBS and incubated for 2 h at 37 °C. After washing, biotinylated F(ab′)2 goat anti-mouse IgG (1:3000) (Jackson ImmunoResearch, West Grove, PA, USA) were added and incubated for additional 2 h at 37 °C following incubation with ExtrAvidin peroxidase conjugate (0.5 μg/ml, Sigma). The reaction was visualized by adding 2,2-azino-bis-(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, 2.5 mg/ml, Sigma) in 10% diethanolamine buffer containing 0.0025% $H_2O_2$. The absorbance was recorded in a spectrophotometer Spectra MaxPlus at 405 nm. The optical density (OD) values registered were related to the OD values obtained from normal (naive) mouse sera.

*Statistical analyses.* Values are expressed as mean ± SEM. Values of $P < 0.05$ were considered statistically significant. Statistical tests used were Mann–Whitney, paired *t*-test, unpaired *t*-test.

## Results

### Histamine levels in synovial fluids and sera of patients with RA and of healthy controls

Circulating levels of histamine from patients with RA was significantly lower compared with those of healthy subjects (0.93 ± 0.16 ng/ml versus 1.89 ± 0.45 ng/ml, $P = 0.0005$) (Fig. 1). Interestingly, paired comparison of histamine levels in the blood and the synovial fluid of RA patients showed that in the joints, histamine levels were even lower (0.93 ± 0.16 ng/ml versus 0.37 ± 0.06 ng/ml, $P = 0.0006$). In contrast, there were no significant differences regarding histamine levels in SF of RA patients and of controls with non-inflammatory joint disease (0.37 ± 0.06 ng/ml versus 0.53 ± 0.12 ng/ml, ns). Comparison of circulating histamine levels of RA patients with erosive (0.92 ± 0.22) versus non-erosive (1.01 ± 0.22) RA, demonstrated that patients with erosive RA had somewhat lower levels of histamine in their blood compared with patients with non-erosive RA (not significant).

### Changes of histamine levels during anti-TNF-α treatment

To assess the effect of anti TNF-α treatment on histamine blood levels, samples were collected from 10 RA patients prior to the first infusion of infliximab and following 40 weeks of treatment (a total of five infusions of infliximab). The levels of histamine in circulation before and after the sixth anti-TNF-α treatment are shown in Table 2. Treatment led to a significant increase of histamine levels in the blood (0.60 ± 0.07 ng/ml vs. 1.10 ± 0.14 ng/ml, $P = 0.005$) (Fig. 2). The increase in histamine levels was inversely correlated with the DAS score ($r = -0.48$) and CRP levels ($r = -0.42$) (Fig. 3A,B). The measurement of circulating IL-10 levels before and after the anti-TNF-α treatment did not display any significant differences. Single infusion of infliximab had no obvious effect on histamine levels (mean: 0.91 ng/ml before and 1.04 ng/ml, after infusion (not significant).

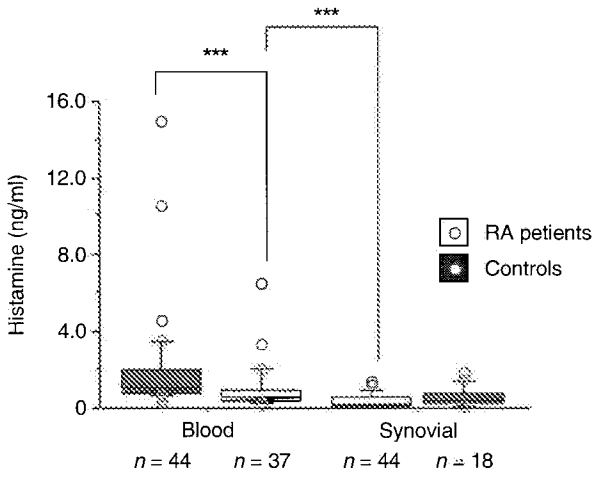


**Figure 1** Histamine levels in the blood and in the synovial fluid of RA patients and controls. ***$P < 0.001$, synovial fluid versus blood in RA patients. ***$P < 0.001$, blood of RA patients versus blood of healthy blood donors.

### Does histamine modulate inflammatory responses intra-articularly?

The histological analysis of mouse knee joints injected with 0.01–10 ng ($n = 24$) of histamine revealed no signs of synovitis, such as intra-atricular influx of leukocytes or pannus formation. To asses if histamine eliminates inflammatory responses, we injected histamine in combination with high mobility group (box chromosomal) B1 protein (HMGB1). HMGB1 triggers the development of arthritis when administered to healthy joints [30–32]. Peptidoglycan, a component of the Gram-positive bacterial cell wall is also known to induce inflammatory response in healthy joints [33–34]. Histamine (10 ng) was co-administered to the joint cavity together with either HMGB1 (2 μg, $n = 16$) or with peptidoglycans (10 or 50 μg, $n = 16$) did not significantly affect the inflammatogenic properties of these molecules. The frequency and severity of joint inflammation in mice injected with histamine/peptidoglycan mixture was similar to those found in the mice injected with peptidoglycan alone (73% versus 82%). The frequency of joint inflammation in mice injected with histamine/HMGB-1 mixture was somewhat higher than in mice injected with HMGB-1 alone (73% versus 36%) (ns).

### Does stabilization of mast cell membrane affect *in vivo* inflammatory responses?

Treatment with SC had no significant effect on degree of paw swelling triggered by olive oil. Indeed no differences in paw thickness were detected between naive and SC-trea-

**Table 2** Impact of infliximab treatment on circulating histamine levels ($n = 10$).

| | DAS28 | Histamine (ng/ml) | IL-10 (ng/ml) | CRP | WBC ($10^9$/ml) | Platelets ($10^9$/ml) | Hb (g/l) |
|---|---|---|---|---|---|---|---|
| Before infliximab | 5.3 ± 1.1 | 0.6 ± 0.2 | 8.1 ± 1.4 | 52 ± 51 | 9.0 ± 4.3 | 355 ± 164 | 124 ± 13 |
| After five infusions | 3.8 ± 1.2 | 1.1 ± 0.2 | 8.4 ± 3.1 | 17 ± 16 | 7.0 ± 2.2 | 290 ± 129 | 131 ± 11 |
| *P* | 0.013 | 0.01 | ns | 0.049 | ns | ns | ns |

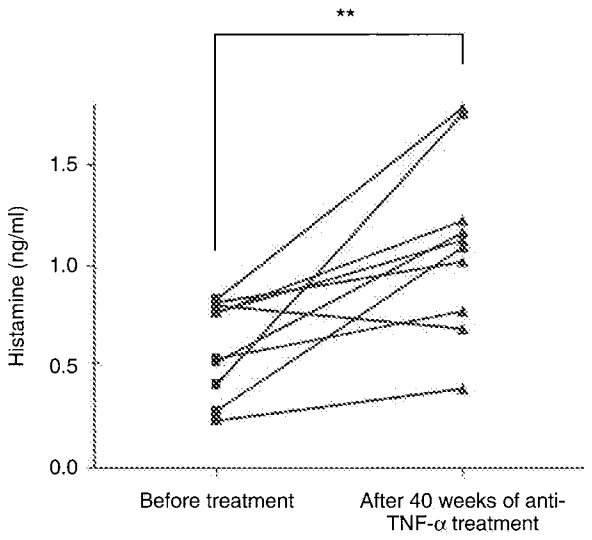


Figure 2 Increase in histamine levels in plasma of RA patients treated with infliximab ($n = 10$). $^{**}P < 0.01$.

ted mice. This indicates that MC inhibition does not prevent granulocyte-mediated inflammatory response. Furthermore, treatment with sodium cromoglycate had no effect on the degree of ear swelling in OXA-sensitized and challenged mice. No difference in ear thickness was detected between control and treated groups, indicating that lymphocyte/macrophage-mediated skin inflammation was not abrogated by MC inhibition. No differences in serum levels of anti-oxazolone antibodies were detected between the sodium cromoglycate treated and control groups.

### Does histamine affect production of pro-inflammatory IL-6?

Mononuclear spleen cell cultures ($n = 3$) were exposed to the increasing concentrations of histamine (0–100 μM) in the presence and absence of T-cell mitogen Con A or superantigen TSST-1. Supernatants were collected after 72 h and an analysis of IL-6 levels was performed. Treatment of splenocytes with histamine alone did not induce production of IL-6. TSST-1 and Con A-induced production of IL-6 (55 ± 4 pg/ml and 80 ± 15 pg/ml). This production was not affected by the presence of histamine in the culture medium (Table 3).

## Discussion

The aims of our study were to: (a) analyse the relationship between histamine levels in the SF and the blood of RA patients; (b) evaluate the impact of a TNF-α inhibition on histamine levels in RA patients; (c) determine whether the histamine molecule itself induces inflammation in the joint cavity of healthy recipients; and (d) assess if histamine has immunomodulatory properties.

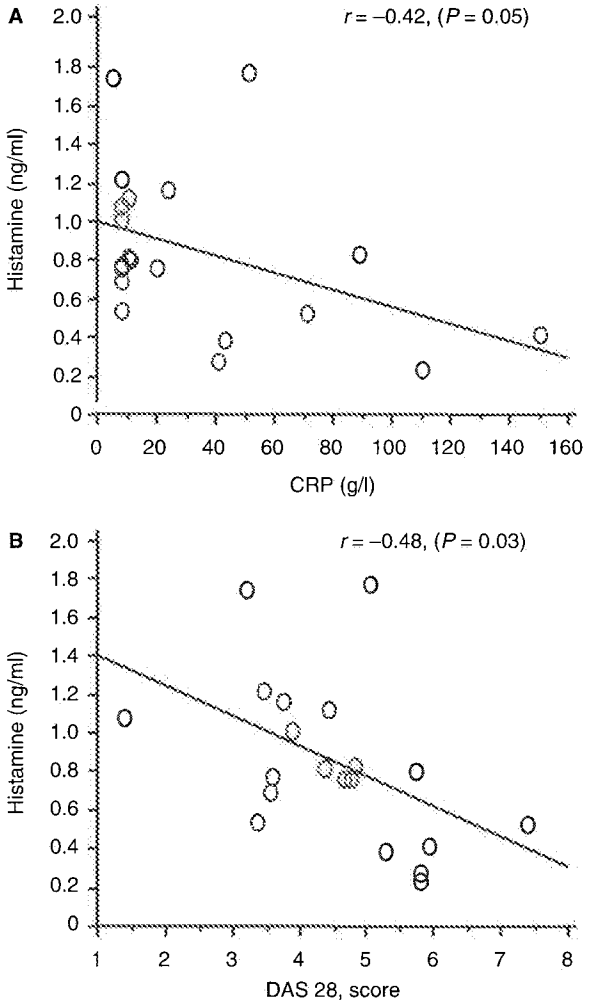


Figure 3 Circulating histamine levels of RA patients treated with infliximab are inversely related to CRP levels (A), and to the disease activity score (B).

Table 3 IL-6 production by mouse spleen cells ($n = 3$), after treatment with different concentrations of histamine.

| Histamine (μM) | Con A | TSST-1 | Mock |
|---|---|---|---|
| 100 | 63 | 48 | 16 |
| 10 | 47 | 52 | 12 |
| 1 | 108 | 55 | 13 |
| 0 | 80 | 55 | 12 |

The SD of Con A (histamine 0 μM) was 15 pg/ml while that of TSST-1 was 4 pg/ml.

Our present data point to the fact that, within the joint cavity, histamine is actively consumed as its levels are significantly lower in the joint fluid than in the circulation of RA patients, despite the fact that the synovial cells are able to actively synthesize this molecule *in situ*. The idea of histamine consumption during inflammation

 

in RA patients is also supported by the observation of low histamine levels in circulation compared with healthy blood donors. We prospectively measured histamine levels in the blood of RA patients undergoing anti-TNF-α treatment. TNF-α promotes inflammatory responses that are important in the pathogenesis of RA and studies have proved that blocking TNF-α improves the course of RA [13, 35, 36]. An analysis of various inflammation markers in 10 RA patients showed that clinical improvement of symptoms was linked to the increased histamine levels in the majority of the RA patients undergoing anti-TNF-α treatment (Fig. 2). This indicates that TNF-α inhibition leads to a significant increase in histamine levels in the blood of RA patients, suggesting that histamine is more likely to be connected to the halting of the disease process in RA, on the contrary to suggestions from most previous studies.

Injection of histamine *per se* did not cause synovitis in healthy recipients. The doses of histamine were chosen based on our *ex vivo* findings in human joints. To ensure broad spectrum of histamine concentrations, up to 1000-fold differences of its intra-articular amount were used. Although histamine usually elicits strong inflammatory response, its effects are typically short lived. The outcome observed could possibly be attributed to short biological half-life of histamine [37]. Given that histamine alone failed to induce an inflammation, we combined histamine with two known pro-inflammatory substances, HMGB-1 [30, 32] and peptidoglycan [1, 34]. The question was whether histamine could affect the HMGB1/peptidoglycan-induced inflammation. Our results suggest that histamine does not influence the inflammatory effects of HMGB1 and peptidoglycan in the joint cavity.

IL-6 is a pro-inflammatory cytokine that is known to be present in high levels in the SF from RA patients [2]. It is produced by many cell types and is thought to play an important role in RA [38]. In our study, exposure of leukocytes to histamine did not have any effect on the production of IL-6. It has been shown that histamine is capable of inducing a dose-dependent IL-6 production in endothelial cells and the observed difference in results may be due to the different cell type used in our study.

Use of SC, an MC stabilizing agent, did not affect *in vivo* cell-mediated inflammatory responses. Stabilization of MC is believed to decrease their degranulation activity and thereby histamine release. SC has also been successfully used to determine the role of MC in a collagen II-induced arthritis model in mice [6, 39]. The inefficacy of SC in the oxazolone-induced DTH reaction was surprising, given that both early and late components of this reaction are dependent on MC [29, 40]. Equivalent ear swelling responses have been found in MC-deficient and wild-type mice [41–42], although strongly reduced ear swelling in MC-deficient animals has also been observed [29]. Route of delivery could potentially have affected the outcome of our experiment, as different methods have been used in various studies. However, our results are in agreement with one study that also examined the effect of SC on DTH reactions and came to the conclusion that it does not alter the responses significantly [43]. Administration of SC *in vivo* did not decrease the olive oil-induced inflammation. Indeed, this type of inflammation operates through the activation of neutrophils and is independent of MC and T cells [27]. In the setting of RA, we believe that properties of histamine may be mediated through its actions on neutrophils. Neutrophils release a range of proteases, hydrolases and also have the ability to produce a series of reactive oxygen intermediates (ROI), therefore having a great capability to inflict damage and cartilage degradation. It has been shown that primed and activated neutrophils in RA synovial fluid secrete above enzymes and ROI within the joint [36]. Histamine inhibits the chemotaxis of neutrophils and production of their cytokines, such as IL-1, IL-12 and TNF-α [20, 24–26, 44]. By binding to H2 receptors, histamine also inhibits phagocytic NADPH oxidase activity causing a decreased production of oxygen radicals [45]. Therefore, we suggest that histamine blocks the production of neutrophil-derived ROI, thus preventing tissue degradation in the joint. Our results show that histamine levels in the blood rise after the treatment and this might suggest that the beneficial action of TNF-α antagonists is mediated through the histamine-dependent inhibition of neutrophil extravasation.

Altogether our study points out that: (a) histamine levels are significantly suppressed in RA; (b) efficient immunomodulatory treatment reverses this suppression. These findings together with other well-known properties of histamine suggest that histamine is anti-inflammatory rather than pro-inflammatory in the setting of human RA.

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